DNA-BASED DETECTION OF FOOD ISOLATES OF *BACILLUS CEREUS* AND ITS BEHAVIOURAL PATTERN IN SELECTED FOODS

The Thesis
submitted to the Department of Studies in Microbiology, University of Mysore in fulfillment of the requirements for the Degree of Doctor of Philosophy
in
MICROBIOLOGY

Ph.D. Candidate
Mr. Desai Shivalingsarj Vijaykumar, M.Sc.

Research Guide
Dr. M.C. Varadaraj, Ph.D.

Human Resource Development
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE
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April 2010

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FORMAT OF PRESENTATION

4.1 PREVALENCE OF POTENT TOXIGENIC / PATHOGENIC ISOLATES OF *BACILLUS CEREUS* IN INDIAN TRADITIONAL FOODS

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MYSORE – 570 020

April 2010

Dedicated

to my

Grandparents

CERTIFICATE

I, **Mr. Desai Shivalingsarj Vijaykumar** certify that this thesis is the result of research work done by me under the supervision of **Dr. M.C. Varadaraj** at **Human Resource Development, Central Food Technological Research Institute, Mysore 570 020**. I am submitting this thesis for possible award of Doctor of Philosophy (Ph.D.) degree in **Microbiology** of the University of Mysore

I further certify that this thesis has not been submitted by me for award of any other degree / diploma of this or any other University.

.....
Signature of Doctoral Candidate

Signed by me on April 08, 2010

.....
Signature of Guide

Date: April 08, 2010

Counter signed by

.....
Signature of Chairperson / Head of Department /
Institution with name and official seal

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FT/HRD/Ph.D./2010 April 08, 2010

CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"DNA-BASED DETECTION OF FOOD ISOLATES OF *BACILLUS CEREUS* AND ITS BEHAVIOURAL PATTERN IN SELECTED FOODS"** submitted by **Mr. Desai Shivalingsarj Vijaykumar** for the award of degree of **DOCTOR OF PHILOSOPHY IN MICROBIOLOGY** to the **UNIVERSITY OF MYSORE**, is the result of the research work carried out by him in the Department of Human Resource Development, Central Food Technological Research Institute, Mysore under my guidance and supervision during the period of Ph.D. programme. This has not been submitted earlier, either partially of fully for any other degree or fellowship.

(M.C.VARADARAJ)
Research Guide

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April 08, 2010

DECLARATION

I hereby declare that the thesis entitled **"DNA-BASED DETECTION OF FOOD ISOLATES OF *BACILLUS CEREUS* AND ITS BEHAVIOURAL PATTERN IN SELECTED FOODS"** submitted to the **UNIVERSITY OF MYSORE**, for the award of degree of **DOCTOR OF PHILOSOPHY IN microbiology**, is the result of the research work carried out by me under the guidance of **Dr. M.C. Varadaraj**, Head, Human Resource Development, CFTRI, Mysore during the entire tenure of my CSIR-Research Fellowship programme. I further declare, that the results presented in this Thesis have not been submitted for the award of any other Degree or Fellowship.

(DESAI SHIVALINGSARJ VIJAYKUMAR)

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Desai, S.V.

Thesis Synopsis

Introduction

Review of Literature

Object & Scope of the Study

Experimental Plan, Results and Discussion

4.1 PREVALENCE OF POTENT TOXIGENIC / PATHOGENIC ISOLATES OF *BACILLUS CEREUS* IN INDIAN TRADITIONAL FOODS

4.2 THERMAL INACTIVATION PROFILE OF VEGETATIVE CELLS AND SPORES OF *BACILLUS CEREUS* SUBJECTED TO SIMULATED TIME-TEMPERATURE COMBINATION OF INDIAN TRADITIONAL FOODS

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Summary and Conclusion

Bibliography

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ATTENDANCE CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"DNA-BASED DETECTION OF FOOD ISOLATES OF *BACILLUS CEREUS* AND ITS BEHAVIOURAL PATTERN IN SELECTED FOODS"** submitted by **Mr. Desai Shivalingsarj Vijaykumar** for the award of degree of **DOCTOR OF PHILOSOPHY IN MICROBIOLOGY** to the **UNIVERSITY OF MYSORE**, is the result of the research work carried out by him in the Department of Human Resource Development, Central Food Technological Research Institute, Mysore under my guidance and supervision during the period of Ph.D. programme.

(M.C.VARADARAJ)
Research Guide

DNA-BASED DETECTION OF FOOD ISOLATES OF *BACILLUS CEREUS* AND ITS BEHAVIOURAL PATTERN IN SELECTED FOODS

Synopsis of the Ph.D. Thesis
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April 2010

From:
Mr. S.V. Desai
CSIR-RF, Dept. of Human Resource Development
CFTRI, Mysore 570 020

April 09, 2010

To:
The Registrar (Evaluation)
University of Mysore
Crawford Hall, Mysore

Dear Sir,

Sub: Submission of Ph.D. Thesis
Ref.: Ex 9.2/Ph.D./DSV/160/2005-06 dt. 27.01.2006
Ex 9.2/Ph.D./DSV/2005-06 dt. 30.11.2009

With reference to the above-mentioned subject, I am hereby submitting the Ph.D. thesis entitled **"DNA-BASED DETECTION OF FOOD ISOLATES OF *BACILLUS CEREUS* AND ITS BEHAVIOURAL PATTERN IN SELECTED FOODS"** along with the duly filled application form and all the necessary documents (enclosed) as shown below for your kind needful.

Thanking you,

Yours faithfully,

(S.V. DESAI)

Encl:
1. Completed application form duly signed by the Guide and Director, CFTRI
2. Fee paid challan (original)
3. Guide's certificate
4. Attendance certificate
5. Copy of Ph.D. registration communication letter
6. Copy of Ph.D. registration extension letter
7. Copy of Master's Degree certificate
8. Xerox copies of all the tuition fee paid challans
9. Synopsis – 15 copies
10. Thesis – 5 bound copies and 1 CD

(M.C. VARADARAJ)
Guide/Head, HRD, CFTRI, Mysore

ABSTRACT OF THE Ph.D. THESIS

Bacillus cereus is an opportunistic foodborne pathogen that contaminates a wide variety of foods. There are mainly two types of illness caused by *B. cereus* in human beings namely diarrhoeal and emetic types. The recent studies reporting the foodborne illness being caused by all the members of *B. cereus* have made the specific identification of *B. cereus* all the more important. The study of the organism has gained significance due to its endospore forming nature, which are resistant to heat, nutrition depletion, desiccation and other such factors. India being a vast and subtropical country with plethora of food varieties being prepared at household and commercial level provides an ideal situation for an organism like *B. cereus* to survive and grow leading to health hazards. In the background of Indian scenario, The present Ph.D. programme attempts to assess the prevalence of toxigenic and non-toxigenic isolates of *B. cereus* in selected foods and understand the behavioural pattern of this bacterial species in culture and food systems.

The native isolates of *B. cereus* obtained from a diverse range of foods revealed the occurrence of *B. cereus* as evidenced by conventional and molecular biology methods. Isolates in varied percent were positive for target genes of 16S rDNA and phosphatidylinositol phospholipase C (*pi-plc*), haemolysin BL (*hbl*) and sphingomyelinase (*sph*). The selected toxigenic food isolates of *B. cereus* revealed a high degree of sequence homology with earlier established selected strains of *B. cereus* and other species of *B. cereus* cluster as evidenced in sequence homology of partial nucleotide sequences of respective PCR amplicons of target genes. Genetic relatedness among selected potent toxigenic native isolates of *Bacillus cereus* based on RAPD-PCR using 4 selected arbitrary primers resulted in a dendogram with 3 clusters covering almost all the isolates except for one isolate, which was of non-cluster type. The cluster patterns could distinguish the isolates of *B. cereus* based on source of isolation, growth temperature and prevalence of toxigenic traits like haemolysin and sphingomyelinase.

The experimental trials undertaken to evaluate the behaviour of vegetative cells and spores of four potent native toxigenic food isolates of *Bacillus cereus* as affected by selected time-temperature combinations used in Indian traditional foods revealed a thermal inactivation pattern, which indicated that the death rate was not constant during the process of heat treatment.

In the present experimental trials, an attempt was made to model the behaviour of vegetative cells and spores of a potent toxigenic native food isolate of *B*. *cereus* CFR 1534 in terms of LPD and GR.

The culture of *B*. *cereus* which exist as vegetative cells and spores have better survival and growth ability in a diverse range of food matrices as a function of temperature, pH and sodium chloride concentration. The generated lag phase duration (LPD) and growth rate (GR) values did reveal the versatility of this native toxigenic food isolate *B*. *cereus*. The model derived in the study was found to be significant and the predicted values were found to be in good agreement for LPD and moderately fitting for GR values. The same native isolate was also studied for its behaviour in two food systems namely (i) a traditional cereal legume based dehydrated nutritious food product based on millet and legume, locally known as *Ragi hurittu* and (ii) a ready-to-use chocolate milk, which is a cocoa-based beverage with reasonable total solids. The bacterial species tend to remain in the product, whether introduced as vegetative cells and/or spores and subsequently, do increase in a slow paced manner. Such a product profile with no visual changes, but harbouring viable population of *B*. *cereus* is of high risk from the viewpoint of causing potential health hazards.

The research findings of this Ph.D. thesis did indicate that the diversified genetic relatedness of toxigenic traits are well spread within *B*. *cereus* cluster and have become stable traits among food isolates of *B*. *cereus* prevalent in the food chain. Being an opportunistic foodborne pathogen, the thermal inactivation pattern and behavioural pattern in culture system and food matrices would enable to develop HACCP protocols in the food chain towards providing microbiologically safe foods to the human population.

Synopsis of Ph.D. Thesis

DNA–BASED DETECTION OF FOOD ISOLATES OF *BACILLUS CEREUS* AND ITS BEHAVIOURAL PATTERN IN SELECTED FOODS

The practices of hygiene and sanitation prevailing in the commercial eateries provide ample scope for contamination with pathogenic bacteria. Among the various bacterial pathogens, strains of *Bacillus cereus* are of significance as they are opportunistic organisms, which can dominate, in any given situation. While many species of *Bacillus* are known for spoilage of foods, strains of *B. cereus* are significant as causative organisms of foodborne illness. *Bacillus cereus* is a Gram positive, rod shaped, endospore-forming bacteria found ubiquitously. The study of *B. cereus* in relation to food has gained significance in the light of its ability to form heat resistant endospores and capacity to grow and produce toxins in a wide variety of foods.

Bacillus cereus causes two types of illnesses in humans namely diarrhoea and emesis. Nevertheless many incidences of *B. cereus* go unreported owing to mild symptoms of self-limiting nature of illness and short recovery periods. The diarrhoeal syndrome is usually caused by several of the enterotoxins, which, in general, are heat labile. As it stands, three types of heat labile enterotoxins involved in food poisoning have been described and well characterized at the molecular level. The emetic syndrome is caused by a single heat stable peptide toxin called cerulide. The ability of cultures of *B. cereus* to grow and produce these toxins is influenced by a set of extrinsic and intrinsic factors. Besides, molecular diagnostic PCR methods have been described for the detection of potent toxigenic cultures of *B. cereus*.

Considering the ubiquitous nature of *B. cereus* in almost all types of foods, the dual phase of existence as vegetative cells and spores, favourable climatic conditions of our country, the prevailing practices of hygiene and sanitation, the varied geographical distribution of isolates and their pathogenicity, there is ample scope to study the

toxigenic potential of food isolates of *B. cereus* through DNA-based methods and also develop models which would predict the potential of organisms to render foods unsafe for human consumption. In this background, the objectives for the present Ph.D. programme were proposed as follows:

- Diversity of isolates of *B. cereus* in selected foods with a focus towards PCR-based detection and analysis for toxin potential
- Extrinsic and intrinsic factors influencing the growth of *B. cereus* (vegetative cells and spores)
- Behavioral pattern of spores and vegetative cells of *B. cereus* in cereal-legume based and milk based foods towards development of predictive models

The Ph.D. thesis has a brief introduction to the focused objectives of study in the background of microbial food safety and the relevance of foodborne pathogenic bacterial species included in the present study. The introduction is followed by a comprehensive literature of status which has attempted to document published relevant research findings covering aspects of (i) relevance of foodborne pathogens, (ii) future of foodborne diseases from a societal perspective, (iii) history and public health significance of *B*. *cereus*, (iv) incidence and outbreaks of *B*. *cereus* food poisoning, (v) taxonomy and characteristics of *B*. *cereus*, (vi) isolation and detection protocols for *B*. *cereus* from foods, (vii) incidence of *B*. *cereus* in a diverse range of foods, (viii) behavioural pattern of *B*. *cereus*, (ix) molecular basis for diversity of pathogenic/virulence traits in *B*. *cereus* and (x) *B*. *cereus* in present scenario of food safety.

In the background of object and scope of study being presented, the main research programme is presented as experimental methods, results and discussion under the following titles:

1.0 PREVALENCE OF POTENT TOXIGENIC ISOLATES OF *BACILLUS CEREUS* IN INDIAN TRADITIONAL FOODS

Considering the documented incidences of *B*. *cereus* in foods in several other countries, in this study, the prevalence of *B*. *cereus* was assessed by conventional selective plating protocol from a diverse range of foods, which included processed rice-based foods with added spices and vegetable salads (traditional fast foods), ice cream, raw milk, traditional milk-based sweets, processed wheat-based foods and spiced cooked rice-based foods. The mean viable population ranged from a minimum of 3.4 $\log_{10}$ CFU/g in case of milk-based foods to a maximum of 5.1 $\log_{10}$ CFU/g in spice-based traditional fast foods. A total of 65 food samples were analyzed and 26 isolates were identified to *B. cereus* group based on morphological, cultural and biochemical characteristics. The growth ability of a few of the native isolates to grow at low and high temperatures showed the potential of native cultures to grow under fairly diverse range of temperatures. PCR detection with species specific Phosphatidyl-inositol specific Phospholipase (Pi-PLC) primers resulted in 12 isolates (46%) out of 26 to be confirmed as *B*. *cereus*. Further, the identity of these isolates was confirmed to their species by 16S rDNA amplification. Detection with primers targeted for haemolysin and sphingomyelinase genes resulted in 8 and 6 isolates being positive, respectively. Approximately, 50% of the isolates exhibited discontinuous pattern of haemolysis in blood agar plate.

The microbial diversity by genetic analysis of four native food isolates of *B. cereus* (CFR 1529, 1530, 1534 and 1536) was assessed using the resultant partial nucleotide sequences of respective PCR amplicons of target genes (16S *rDNA*, *pi-plc, hbl and sph*) and degree of sequence homology determined. Based on these sequences, Force type Neighbour-joining phylograms were generated to determine the

evolutionary relatedness amongst homologous genes represented in the genome of divergent species from GeneBank database. The phylograms generated and multiple alignment sequences for the respective target genes of *16S rDNA*, *pi-plc*, *hbl* and *sph* revealed high degree of homology (>90%) of the native isolates with other well documented strains of *B. cereus* and other species of *B. cereus* cluster.

All the 12 native food isolates of *B. cereus* were subjected for RAPD-PCR analysis to assess their genetic diversity. The genetic similarity (GS) coefficient for the isolates of *B. cereus* resulting from RAPD analysis ranged from 0.040 (between the isolates CFR 1534 and CFR 1533) to 0.470 (between the isolates CFR 1532 and CFR 1535), except CFR 1540. The dendrogram generated based on GS coefficients of RAPD analysis showed clear distinction into major and minor clusters with the presence of 3 major clusters A, B and C and sub-clusters within the major clusters. The sub-clusters, A1 and A2 had 2 and 4 isolates, respectively, while B1 had 3 isolates and B2 one isolate. Cluster C had only one isolate. The native isolate of *B. cereus* CFR 1540 was not present among the clusters generated and remained as a non-cluster pattern.

2.0 THERMAL INACTIVATION PROFILE OF *BACILLUS CEREUS* VEGETATIVE CELLS AND SPORES SUBJECTED TO SIMULATED TIME-TEMPERATURE COMBINATIONS OF INDIAN TRADITIONAL FOODS

In general, indigenous foods are subjected to heat processing, which on many occasions also get reheated. As *B*. *cereus* is ubiquitous in its habitat, it becomes significant, because of its dual existence as vegetative cells and spores. Moreover, heating stimulates spore germination. It becomes very important to address thermal inactivation profile of *B*. *cereus* in common type of heating menstrum, which simulates a close profile to food matrices and commonly used time-temperature combinations during processing.

The thermal resistance of 3 potent toxigenic native food isolates of *B. cereus* in terms of *D*- and z-values of vegetative cells and spores were determined in saline, brain

heart infusion (BHI) broth, skim milk and whole milk. The *D*-values for the vegetative cells across the different menstra ranged from the lowest of 3.45 min at 60ºC to the highest of 10.6 min at 56ºC in saline. The findings revealed that there was no marked difference in the thermal resistance of vegetative cells of the cultures studied. The mean *D*-values for saline, BHI broth, Skim milk and whole milk were 5.6, 5.5, 5.4 and 5.1 min, respectively. The 12 *D*-values, also termed as commercial sterility values for vegetative cells of *B*. *cereus* cultures ranged from a lowest of 42 min at 58 and 60ºC to a highest of 127.2 min at 56ºC. The z-values of vegetative cells of *B*. *cereus* cultures ranged from 9.3°C for *B*. *cereus* CFR 1521 in BHI broth to 24°C for *B*. *cereus* CFR 1532 in whole milk. The results showed that heating media did not influence as a singular effect on heat inactivation pattern of vegetative cells. The thermal death curve for the vegetative cells in the present study was non-linear indicating that the inactivation rate was not constant, but rather exhibited a sigmoidal shape with shoulder and tailing pattern.

The *D*-values of spores ranged from a lowest of 4.4 min at 95°C to a highest of 19.45 min at 85ºC. Further, mean *D*-values obtained for the spores in different heating menstra like saline, BHI broth, skim milk, and whole milk were 9.6, 8.9, and 7.3 and 9.6 and min, respectively. The survival curves obtained for spores of isolates of *B*. cereus tested in the present study showed a curvilinear pattern with shoulder (lag phase) followed by a linear declining pattern which showed that the inactivation pattern did not follow first order kinetics. The z-values of the spores of isolates of *B. cereus* ranged from 16.6 to 38.4°C.

3.0 INFLUENCE OF CULTURAL AND NUTRITIONAL ATTRIBUTES ON THE BEHAVIOURAL PATTERN OF *B. CEREUS* (VEGETATIVE CELLS AND SPORES) IN BROTH SYSTEM

The uniqueness of cultures of *B*. *cereus* has been their ability to survive and also profilerate under varied intrinsic and extrinsic factors. This has been due to the dual

phase of *B*. *cereus*, wherein with the slightest onset of unfavourable conditions, the vegetative cells tend to become spores and tide over all adverse conditions. Similarly, the shift towards favourable conditions would enable the spores to germinate in to vegetative cells, which then bring about all the undesirable traits in foods. These attributes provide an opportunity to analyze selected cultures and nutritional parameters that would influence the behaviour of cultures of *B*. *cereus* in the background of source of the cultures as well as their phenotypic and genotypic characteristics.

The growth behaviour of the vegetative cells and spores of a potent toxigenic native isolate of *B*. *cereus* CFR 1534 in terms of lag phase duration (LPD) and growth rate (GR) was studied. The experimental LPD values for vegetative cells ranged from 3.13 to 31.3 h. A fairly high degree of correlation coefficient was observed between experimental and predicted values. Analysis of correlation coefficients showed that temperature had an influencing effect on the LPD values. The experimental GR values ranged from 0.2 to 2.2/h. The pH of the medium was found to have major influence on the GR of the culture. The response surface plots revealed that at pH levels of 5.5 and 6.5 LPD values with increasing concentrations of NaCl from 2 to 6%. However, at pH 7.5, with increasing NaCl concentrations, the LPD values showed an increasing trend, although initially at 2 and 2.5% levels of NaCl, negative LPD values were observed. In contrast, the plots derived for GR values had a varied pattern at the 3 pH levels of 5.5, 6.5 and 7.5.

The experimental LPD values for spores ranged from 5.85 to 20.5 h. Temperature appeared to be the influencing factor for the LPD of spores of *B. cereus* CFR 1534. The experimental GR values ranged from 0.24 to 0.73/h. The lowest GR was recorded in growth conditions of 22°C temperature, pH of 6.5 and NaCl level of 4% and the highest value at temperature of 32°C, pH of 6.5 and 4% NaCl level. It could be visualized from the response surface plots generated for the behaviour of spores that at

all temperatures of the experimental design, the LPD values progressively increased with increasing levels of NaCl. The plots for GR values were of interest in that the trend in behaviour was an increase from the initial GR values at 2-3% NaCl and at incubation temperatures of 22-42°C, after which at levels of 3.5-6.0% of NaCl, the GR values progressively decrease.

The findings enabled in assessing the quantitative effect and synergistic interaction of the three influencing factors (temperature, pH and salt concentration) on the LPD and GR of the selected native isolate of *B*. *cereus* CFR 1534.

4.0 BEHAVIOURAL PATTERN OF *BACILLUS CEREUS* IN SELECTED FOOD MATRICES

The cultures of *B*. *cereus* are known to be prevalent under varied conditions encountered in the food chain. It is well documented that this bacterial species has a very close affinity with cereal and legume-based food commodities, followed by milk and milk products and other foods. Often, cereals and legumes are used as nutritious foods primarily as dehydrated premixes for further use based on consumption needs. Invariably, in the traditional methods of preparation (pre-processing) of such foods and subsequent storage and use (post-processing), there occurs ample opportunities for contamination with *B*. *cereus*. Being a dehydrated product, the focus would be on spores as they can survive under adverse conditions (low water activity) for longer storage periods. In a similar manner, many heat processed ready-to-use foods are also open to chance contamination with *B*. *cereus*, which may be due to faulty / under-processing or post-processing weak linkages in the food chain.

The behavioural pattern of vegetative cells and spores of a potent toxigenic native isolate of *B*. *cereus* CFR 1534 as a function of temperature, initial inoculum and storage period was studied using Fractional Factorial Design in 2 food systems namely

(i) a traditional cereal legume based dehydrated food product, locally known as *Ragi hurittu* and (ii) a ready-to-use chocolate milk.

During storage at 20 and 30°C of *Ragi hurittu*, initially spiked with population of 3.3 $\log_{10}$ CFU/g of vegetative cells, there was an increase of almost 2.0 logs in the viable count in 2 d of storage, after which the numbers remained same till 6 d. No appreciable increase in counts was observed during the storage period of those samples spiked with initial levels of 5.3 and 7.3 $\log_{10}$ CFU/g. A similar trend of no change in counts was observed at 40°C storage. With an initial inoculum of 7.3 $\log_{10}$ CFU/g and at all 3 temperatures, a marginal decrease was observed in the viable population of *B*. *cereus*. A reasonable degree of correlation coefficient was observed between experimental and predicted values. In the case of spores of *B*. *cereus* CFR 1534, with the initial level of 3.3 $\log_{10}$ CFU/g, no change in the counts was observed during storage period. A decrease in the counts was observed with initial levels of 5.3 and 7.3 $\log_{10}$ CFU/g. The response surface plots generated revealed an identical behavioural pattern with that of experimental trials.

In a quite contrasting product profile, which happens to be chocolate milk, the vegetative cells and spores of *B*. *cereus* introduced at the 3 initial levels showed a marginal increase in viable population during the storage period at defined storage temperatures. The relationship between experimental and predicted values was in close agreement and the primary influencing factor was the initial inoculum. The response surface plots for vegetative cells revealed a slow paced increase in population levels during storage period, while those generated for spores showed a marginal decrease in counts in initial 2 d storage, followed by a slow paced increasing pattern.

In view of the changing pattern in viable population of *B*. *cereus* during storage, pH of chocolate milk decreased from an initial level of 6.4 to 4.5. This led to increase in acidity and consequently visual signs of spoilage like off flavor and thickening of product.

On the other hand, the dehydrated product (*Ragi hurittu*) revealed no change in the visual appearance of product as well as no off flavours and product consistency.

The research findings of present study have been briefly summarized along with a viewpoint about microbial food safety aspects being presented under Summary and Conclusion, bringing into focus the highlights of individual experimental trials. The findings did indicate that it becomes highly complicated to eliminate *B*. *cereus* uniformly in all the food environments, as it was amply established in two different food matrices. The microbial diversity could be well visualized through the application of molecular biology methods, wherein almost 90% homology does exist in the genetic make-up of the strains of *B*. *cereus* as well as there occurs a close relationship with other species of the cluster.

The thesis concluded with the documentation of details of relevant references cited in the running text of thesis in an alphabetical order for better reading and tracing of cited references.

S.V. Desai
Research Student

M.C. Varadaraj
Research Guide

1.0 INTRODUCTION

The understanding of biological principles of microflora associated with plants and animals in their natural habitats is of great significance, since human food sources are basically of plant and animal origin. Food is considered as the fuel of life. It is the fundamental source of energy for all human beings. These foods are complex systems. Ecosystems are composed of the environment and the organisms that dwell in this habitat. The food environment consists of intrinsic factors inherent to the food (e.g., pH, water activity and nutrients) and extrinsic factors external to it (e.g., temperature, gaseous environment and competitive microflora). Foods can be heterogenous and the heterogeneity can be associated with gradients of both, intrinsic and extrinsic factors.

Foods occur in diverse forms comprising mainly of milk and milk products, cereal and cereal products, fruits and vegetables, meat and meat products, sea foods, sugar and sugar products. The food with its nutrients, apart from being consumed by humans, also acts as an excellent media for the growth of spoilage and pathogenic microorganisms. A number of factors contribute to food being unsafe and causing foodborne illness. They could be summarized as follows:

- Inadequate control of temperature during cooking, cooling and storage
- Poor personnel hygiene and sanitary practices
- Cross-contamination of raw and processed products
- Inadequate monitoring of processes

It is presumed, that right from the beginning, the problems of spoilage and food poisoning were encountered. In order to overcome many of these problems as well as increase the shelf life of foods, a majority of the foods are either canned or dehydrated or frozen or fermented or irradiated or chemically preserved. All these contributing factors make food microbiology a many-faceted and challenging area of scientific developments.

Foodborne diseases are syndromes that are acquired as a result of ingesting foods that contain either an infectious or a toxigenic microorganism or a poisonous metabolite produced by these organisms. For convenience, they are classified into three categories namely (I) infections, (ii) toxin-mediated infections and (iii) poisoning or intoxications. The infections occur when microorganisms invade and multiply in the intestinal mucosa or other tissues. The toxin-mediated infections are caused by certain pathogenic bacteria that produce enterotoxins (substances that effect balances of water, glucose and electrolyte transfer in the host) during their colonization and growth in gastro-intestinal tract. The poisoning or intoxication is the result of ingestion of foods which contain preformed poisonous substances or toxins elaborated by microorganisms. A foodborne disease or an outbreak is defined as an incident in which two or more persons experience a similar illness. Epidemiological studies have indicated food and water as main causative agents of food poisoning outbreaks. Foodborne diseases are a continuing problem of high magnitude in all the countries, both developed and developing. In developing countries, a significant proportion of deaths and illnesses are attributed to gastro-enteritis, which are mainly foodborne.

The study of disease causing microorganisms associated with food forms an important and continuing topic of microbiology. The study addresses safety aspects of food in relation to human beings. This food safety is evaluated in terms of acceptable level of risks. The changes in food processing technologies and consumer preferences have led to an increased consumption of minimally processed foods. As awareness about food safety issues is increasing and there is a need for the countries to provide greater assurance about the safety and quality of foods. Food safety problems evolve with changes in society, economy, lifestyle and eating habits.

The changes in the types of food consumed, the geographic origins of food products and the ways in which different foods are processed affect both the

potential for contamination and the adequacy of safety measures employed. Food safety can be enhanced by systematically concentrating upon minimizing opportunities for contamination at all stages in the food chain. In this background, there is a growing recognition for application of microbiological analytical tools to address problems related to food safety. Although methods like pasteurization, refrigeration and other unit operations being in use through the approach of hazard analysis critical control points have significantly improved the safety of the foods from a commercial view point, foodborne illness still remains a major cause of morbidity and mortality. Foodborne illness occurs when a person contracts disease by eating food that has been contaminated with a undesirable microorganism or toxin. Many cases of foodborne illness go unreported because of mild symptoms and self-limiting nature.

Enumeration / detection and isolation of foodborne pathogens from the samples are often difficult due to the high number of contaminating and indigenous microflora and a low number of the target pathogen. The conventional methods of isolation, which includes pre-enrichment and selective enrichment, have moderate sensitivity and specificity. They are laborious and time consuming and have the limitation of ambiguity in identification of the microorganisms. Advances in molecular biology and genetics have revolutionized the field of analytical food microbiology. Polymerase chain reaction (PCR) is an extremely powerful tool that enables exponential amplification of a specific sequence in short time. PCR assay can be designed in a multiplex pattern to simultaneously detect several genes. The sensitivity and specificity of the technique combined with less time intensive factor has made it a method of choice for detection and identification of microorganisms. Despite the identification of species it is observed that differences at the strain level exists and thereby reflect different degrees of diversity at molecular level. An insight into the diversity of the strains and their clustering helps in understanding the geographical distribution and clonal lineage of the strains involved. This approach of

epidemiological study is significant from a clinical perspective which helps in diagnosis and treatment in case of outbreaks.

The food industry with its diverse range of products is governed by strict food legislation. In the present global scenario, microbiological safety has become a very key issue with emphasis on foodborne pathogenic bacteria. The important bacterial genera known to play a predominant role either in bringing about spoilage of foods or causing health hazards due to the production of toxic metabolites are as follows: *Aeromonas hydrophila*, *Bacillus cereus*, *Campylobacter jejuni*, *Citrobacter* sp., *Enterococcus* sp., *Erwinia* sp., *Escherichia coli*, *Listeria monocytogenes*, *Micrococcus luteus*, *Proteus vulgaris*, *Pseudomonas* spp., *Salmonella* spp., *Serratia* spp., *Shigella* spp., *Staphylococcus aureus*, *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Yersinia enterocolitica*.

Bacillus cereus is known to be a heat resistant organism mainly due to its endospore forming nature. The thermal resistance of the organism is critical for the food safety. Strains which are resistant to heat can survive heat treatment during food processing and continue to be cause of concern. Moreover the thermal resistance is known to vary for different media and strains. In this context, the thermal tolerance of selected strains of native isolates has been studied in different media. The determination of the heat resistance of the native strains would give an understanding of the potential of the native strains to withstand the thermal processing conditions.

Microbial growth in foods is a complex process governed by genetic, biochemical and environmental factors. Predictive microbiology through the use of mathematical models has enabled to study the behaviour of the microorganisms of concern under the influence of key factors in the food environment, build a cumulative store of knowledge and to develop the means to interpolate calculated microbial responses. The developments in the microbial modelling are contributing to a considerable expansion in the number of computerized databases and equations

necessary to create predictive microbial models. Microbial modelling is the use of mathematical expressions to describe the behaviour of a microorganism. This includes pattern that show the changes in bacterial populations with time and the rate of change as influenced by different environmental factors. The modeling of microbial populations has resulted in coining of the term 'Predictive Food Microbiology', which has emerged as a major area of research. Mathematical modelling for the behaviour of foodborne microorganisms was initiated in the 1920s with thermal death time calculations.

The survival and growth of these microbial contaminants in foods are influenced by both intrinsic and extrinsic factors. A number of factors such as temperature, pH, salt & sugar concentrations, acidulants, water activity, preservatives and others are found to influence the growth / survival. The bacterial organisms grow best in the pH range of 6.5 to 7.5, although they can survive at extremes of pH 4 and 9. These intrinsic and extrinsic factors are to be considered when assessing whether a microorganism is likely to pose risk factors in food system. The growth responses of the microorganisms from experiments performed according to the laid-out design are systematically recorded and modelled as functions of different parameters each at different levels. The behaviour of the modelled microorganism is then predicted by the multivariate analysis and response surface plots with three dimensional graphs. Models are now a standard research technique and a powerful tool in designing the new formulations of foods and controlling food processes and will be a powerful tool in the quest to optimize quality and safety. While most of the modelling studies have been performed in laboratory growth media, very few have been focused on food systems.

In the growing concern for microbial food safety on a global platform coupled with the advent of mandatory HACCP programs for almost all kinds of foods including those of traditional and/or ethnic foods, the development of more specific and reliable detection methods for toxigenic isolates of *B*. *cereus* is of great significance in public

health microbiology. Several PCR-based markers including primers for species specificity, rRNA sequences and different toxin genes have been evolved to detect for the presence of virulent strains of this bacterial species. It becomes imperative, that the focus of research investigation should aim at assessing the prevalence and identification of toxigenic traits in food isolates of *B*. *cereus*, their thermal inactivation profile in line with those existing in food processing, molecular basis for the diversity of native isolates and predictive models for the behaviour in broth and food systems. These aspects would give a comprehensive approach from the point of food safety and public health perspective leading to the betterment of quality of human life.

2.0 REVIEW OF LITERATURE

2.1 RELEVANCE OF FOODBORNE PATHOGENS

Food being one of the most basic necessities of life has prominent place in the Society. It is evident from the historical studies that the food habits and culture have co-evolved with human civilization and continues to do so even in the present day. Over a period of time, food has undergone divergent ramifications based on the socio-religious, economic, environmental and geographical factors. The choice of food is based on social, cultural, psychological, religious, spiritual and biological factors. Food, apart from being an organic fuel to the living system and gastronomic delight has wider implications in the form of cultural representations of the communities, hospitality gesture, commerce, international linkages and health-driven aspects.

In the present scenario of scientific and technological advancements, food has acquired an unprecedented prominence in terms of nutrition, security and safety. With the advent of globalization, there has been an increase in the consumer awareness with regard to food related aspects in general and food safety in particular. The increase in the number and severity of foodborne outbreaks world-wide has created more public awareness about food safety. Studies into the archeological sites report that, barley flourished between 18300 and 17000 years ago in Nile valley in Egypt and by 3000 B.C., people of Sumer (now Iraq) had developed agricultural economy. It was only in 10^{th} century A.D., that microbiological food poisoning was recognized in civil law (Tannahill, 1973; Wendorf et al. 1979). Food poisoning by spoiled grains was recognized by ancient Greeks and Romans. Epidemics occurred through the middle ages in Europe, Russia and elsewhere. In the middle of the 16^{th} century, epidemics associated with grains infested with *Claviceps purpurea* were reported. By the middle of the first millennium, some sections of population in India had begun to list some items of diet as impure. Rice, which had turned sour through being left to stand overnight, ready-to-eat food from

the market and dishes stained by insects or mice were regarded unfit for consumption (Tannahill, 1973). Spoilage of food and associated diseases are thus a cause of concern since ages.

Since food is biological in nature and is capable of providing nutrients to consumers with nutrients, it is equally capable of nourishing the contaminating organisms. Foodborne disease is any illness that results from ingestion of food. Food can contain microbiological or chemical entities that cause health related complications. These causative agents range from being an inherent constituent of food to inadvertent addition during food production, processing or preparation (Snowdon et al. 2002). Generally, bacterial foodborne illnesses are classified into two different categories namely infections and intoxications. Foodborne infections are caused by ingestion of food containing viable bacteria which can grow, colonize and proliferate in the host. Food intoxication is the consumption of food containing preformed bacterial toxin(s) elaborated by the organism during its growth phase. The most common symptoms associated with foodborne illnesses are diarrhoea, vomiting, nausea and abdominal cramps along with fever. The manifestations may be acute and self-limiting or chronic and life-threatening, depending on the virulence of the causative organism and the health status of the individual.

The public health challenges related to foodborne diseases are changing rapidly mainly because of newly identified pathogens and vehicles of transmission. Several factors contributing to the emergence of foodborne diseases are: changes in human demography and behaviour, technology and industry, international travel and commerce, microbial adaptation, economic development and breakdown of public health measures. The complex sources of our food supply, rapid transport of perishable foods and consumer preferences for new varieties of foods have led to exposure to new pathogens. Increased demand for ready-to-eat and minimally processed foods along

with eat-out culture in restaurants has also contributed to the new epidemiology of the pathogens (www.iom.edu).

Since the last decade, increase in foodborne illnesses have had a significant impact on public health and has become a widely focused area of research. According to a study by World Health Organization, hundreds of millions of people world-wide are affected by foodborne illnesses resulting in hospitalizations and deaths (www.who.int). It is estimated that in U.S. the annual figure is 76 million cases of illness, 3,23,000 hospitalizations per year and 5000 deaths (Mead et al. 1999). Nevertheless, the relative importance of foodborne diseases tends to be under-estimated largely because most of them are seldom fatal, lasts for short duration, self-limiting and share common symptoms such as diarrhoea and vomiting. Further, the public health importance of some pathogens considered emerging is yet to be ascertained. Thus, foodborne illness is a large and complex problem with background information needed on the epidemiology, cost and risk of disease for effective control measures (Snowdon et al. 2002).

The spectrum of foodborne pathogens includes a variety of bacteria, viral pathogens and parasites. There has been a paradigm shift in this spectrum over time, as well established pathogens have been controlled to a great extent, whereby new ones keep emerging. In addition to this, the significant aspect of the pathogens is the emergence or recognition of new pathogens, antimicrobial resistance, and identification of pathogens that are highly opportunistic. New pathogens can emerge, because of dynamic nature of ecology, changing technology that connects a potential pathogen with the food chain or by acquiring the virulence traits from pathogenic strains. Initialization and sustaining efforts with the objective of determining the magnitude of the problem and control measures need to be undertaken at national and international levels.

The pathogen spectrum can change substantially over a period of time. This can happen because the pathogens whose transmission is well understood could be

controlled and other bacterial species may emerge in their place. As a result, the frequency of specific infections may change, indicating a balance between the ecologies that support bacterial community, cultural habitats and technologies that limit contamination. Despite protocols have been established towards control of many foodborne pathogens of public health significance, the risks caused by several pathogens remain still substantial. In this background, several newer pathogens, which have been on a low profile, are likely to continue to be recognized, as a result of which, population that is aging and immuno-compromised will be at higher risk. On the economy front, as a result of contamination of food by pathogens, the risk for the company manufacturing the food is a function of damage it causes to consumers in terms of health hazards. Hence it is necessary to include the medical cost, product recall and economic sanctions that have a bearing on company's exchequer (Domenech et al. 2005).

Till now, 27 organisms are considered as foodborne pathogens and 13 of these, considered emerging are recognized so in the past 25 years and account for the bulk of the burden of foodborne diseases (Tauxe, 2002). In the following paragraphs, a brief description of the major foodborne bacterial pathogens in terms of their public health significance is presented.

2.1.1 *Aeromonas hydrophila*

Aeromonas hydrophila is a Gram-negative and rod-shaped bacterium. It is present in all fresh water environments and in brackish water. Some strains are capable of causing illness in fish and amphibians as well as in humans who may acquire infections through open wounds or by ingestion of a sufficient number of the organisms through food or water. Strains of *A. hydrophila* cause gastroenteritis in healthy individuals and septicemia in immuno-compromised human beings. It may spread throughout the body

and cause a general infection in persons with impaired immune systems. Two distinct types of gastroenteritis have been associated with *A. hydrophila* one, a cholera-like illness with a watery diarrhoea and the other a dysenteric illness characterized by loose stools containing blood and mucus. It is present in fish, shell fish, red meat and poultry. Isolates of *A. hydrophila* can be recovered from most foods by direct plating onto a solid medium containing starch as the sole carbohydrate source and ampicillin to retard the growth of most competing microorganisms. Chloramphenicol and tetracycline are antibiotics of choice for treatment of the disease.

2.1.2 *Bacillus cereus*

Bacillus cereus is a rod-shaped, Gram-positive, endospore forming and facultative anaerobic organism found ubiquitously. The resistance of its spores to adverse environmental conditions has enabled it to get distributed widely. It is normally found in soil, dust and water. Because of its wide presence in the environment, it can be isolated from a variety of foods, both raw and processed. It is invariably found in vegetables, cereal preparations, milk and dairy products, spices, sweets and meat & meat products. Its presence in foods is not a matter of concern unless the organism grows and produces toxins giving rise to foodborne illness. Although *B. cereus* is the most common organism associated with foodborne illness, other *Bacillus* species like *B. licheniformis, B. subtilis, B. pumilus and B. brevis* are also implicated in food poisoning outbreaks. Presence of high numbers of this organism in foods is indicative of the organism's ability to be an opportunistic pathogen (Rajkowski and Bennett, 2003).

The number of outbreaks of *B. cereus* is normally under-reported owing to the mild symptoms and self-limiting nature of the disease. In Japan, emetic type of illness is dominant over diarrhoea, whereas in Europe and North America, the diarrhoeal type is more reported. The incidences are also reported from Iceland, The Netherlands,

Norway, United Kingdom, Bangladesh and Spain. India being a sub-tropical country with a wide variety of food items, the prevailing congenial environment is suitable for the contamination of *B. cereus*. It occurs as a contaminant during both pre-processing and post-processing operations in the food chain.

2.1.3 *Campylobacter jejuni*

Campylobacter jejuni is one of the many species within the genus *Campylobacter* belonging to family Campylobacteriaceae. The organisms are curved, S- shaped or spiral rods of 0.2 to 0.9 µm wide and 0.5 to 5 µm long. They are Gram negative, non-spore forming, motile, microaerobic, susceptible to low pH (2.3) and grow above 30°C. It is zoonotic with many animal reservoirs like rodents, wild birds, sheep, cows, poultry and pets. Contaminated vegetables and shellfish may also act as vehicles (Altekruse et al. 1994). It is prevalent in many uncooked animal foods. Most *Campylobacter* spp. are associated with lower gastro-intestinal tract infection diarrhoea and extra-intestinal infections including bacteremia, urinary tract infection, meningitis and Guillain-Barre syndrome. It is susceptible to a variety of environmental conditions, does not grow or survive well in food and readily killed by heat treatment. The pathogen has a high degree of virulence with a low infectious dose (Blaser, 2000).

2.1.4 *Clostridium botulinum*

Clostridium botulinum is a Gram-positive, spore-forming, rod shaped and obligate anaerobic bacterium that is widely distributed, occurring in soils, fresh water, marine sediments and intestinal tracts of many animals. The main sources of isolation include canned and sealed vegetables, meat and meat products, fish, infant foods and honey. The increase in the consumption of ready-to-eat, minimally processed foods, particularly those packaged in modified atmospheres with reduced or no oxygen enhances the

incidence of botulinum outbreaks. Several outbreaks have been reported in Japan, U.K. Canada and United States. The symptoms comprise nausea, vomiting, followed by neurological disorders, which include visual and respiratory impairments, loss of normal mouth and throat functions, fatigue, and lack of muscle co-ordination. The toxin of *Cl. botulinum* can be fatal to mankind. The control measures include thermal destruction of the spores or inhibition of spore germination and vegetative cell growth (Austin, 2002).

2.1.5 *Clostridium perfringens*

Clostridium perfringens is a Gram-positive, rod-shaped, encapsulated, non-motile bacterium that is known to cause a wide variety of human diseases. The two foodborne diseases caused are (i) *Cl. perfringens* type A food poisoning and necrotic enteritis. The organism is known to produce two toxins namely *Cl. perfringens* enterotoxin (CPE) and beta-toxin. The CPE toxin induces fluid/electrolyte intestinal alterations (Labbe, 1989). It is present ubiquitously and isolated from soil, foods, dust and intestinal tracts of humans and animals.

Meat (mainly beef and poultry) and meat-containing products (gravies and stews) and Mexican foods are considered as main vehicles of foodborne illness caused due to *Cl*. *perfringens*. The symptoms of *Cl*. *perfringens* type A food poisoning are diarrhoea and abdominal cramps observed in about 8 to 16 h after ingestion of contaminated food and then subside within next 12 to 24 h. Vomiting and fever are not the typical features of the illness. The cells are killed when exposed to stomach acidity. Hence, the illness caused develops only when a heavily contaminated food (> 10^6 CFU/g of food) is being consumed. The enterotoxin is produced in the intestines when the isolates sporulate (Labbe and Duncan, 1977).

2.1.6 *Escherichia coli*

Escherichia coli is a Gram-negative, rod shaped, facultative anaerobe in the intestinal tract of humans and warm-blooded animals. Most *E. coli* strains are harmless, but some are pathogenic and cause diarrhoea. Based on virulence characteristics, diarrhoeagenic *E. coli* are categorized into distinct groups which include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and enterohemorrhagic *E. coli* (EHEC). *Escherichia coli* O157:H7 was recognized as a human pathogen in 1982 and is a well studied pathogen due to serious nature of the symptoms like hemorrhagic colitis and hemolytic uremic syndrome along with bloody and non-bloody diarrhoea in comparison to others which cause only mild symptoms. Outbreaks have been associated with the consumption of contaminated high acid foods, which include apple juice, fermented dry salami, raw vegetables and recreational and drinking water in different countries like Canada, Japan, Africa, U.K. and U.S.A (Doyle et al. 2002).

2.1.7 *Listeria monocytogenes*

Listeria monocytogenes is one of six species in the genus *Listeria*. *Listeria monocytogenes* is a Gram-positive, ovoid to rod-shaped, hemolytic organism with characteristic tumbling motility, facultative anaerobe and widespread in the environment. It is an atypical foodborne illness of public health concern due to the severity of the disease (meningitis, septicemia and abortion). Persons with suppressed T-cell mediated immunity are more prone to listeriosis. The outbreaks of listeriosis are associated with vegetable, dairy, sea foods and meat products. Strict maintenance of storage temperature in the distribution chain will reduce the incidence of listeriosis to a large extent (Harris, 2002).

2.1.8 *Salmonella* spp.

Salmonella enterica was first isolated in U.S. in 1885 from swine suffering with hog cholera. *Salmonella* species are facultatively anaerobic, Gram-negative, rod shaped bacteria belonging to the family Enterobacteriaceae. The biochemical identification is generally coupled with serological confirmation involving somatic (O), lipopolysaccharides (LPS), flagellar (H) and the capsular (Vi) antigens (Le Minor, 1981). *Salmonella* spp. is widespread in natural environment. Apart from being reported in fruits, vegetables, spices, chocolate and milk products, it is predominantly present in meat and poultry- based foods, of which eggs remain a significant reservoir. Human *Salmonella* infections can cause enteric (typhoid) fever, uncomplicated enterocolitis and systemic infections by non-typhoid microorganisms. The prevalence of the *Salmonella* in the global food chain and its consequences on human health is of concern and hence need to be controlled.

2.1.9 *Shigella dysenteriae*

Shigella species are Gram-negative rods, non-motile and oxidase negative. There are four species of the genus namely *Shigella dysentriae, S. flexneri, S. boydi and S. sonnei*. The common foods that have been implicated in outbreaks include potato, salad, chicken and shellfish. The organism is not associated with any particular type of food. The clinical features of shigellosis range from a mild watery diarrhoea to severe dysentery. The incubation period for shigellosis is 1 to 7 d, but the symptoms begin within 3 d. The illness is often self-limited. The complications of the disease include severe dehydration, intestinal perforations, septicaemia and haemolytic uremia syndrome (HUS). Human beings are the natural reservoirs of *Shigella* spp. The low infective dose (10^2 to 10^3 CFU/g), oral route of transmission, wide geographical

distribution of the strains and sensitivity of the human population to *Shigella* infection makes this organism a matter of concern in public health (Smith, 1987).

2.1.10 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive coccus with cells occurring in clusters like bunches of grapes and facultative anaerobe. It is widely distributed and isolated from milk and milk products, eggs, bakery products, meat and meat products, poultry and vegetable salads. It is ubiquitous in nature and predominantly humans and warm-blooded animals act as primary reservoirs. Staphylococcal food poisoning is an intoxication resulting from the ingestion of food containing one or more preformed staphylococcal enterotoxins. The symptoms include diarrhoea, vomiting, nausea, retching and abdominal cramps. Many cases go unreported due to the mild and self-limiting nature of the illness (Wong and Bergdoll, 2002).

2.1.11 *Vibrio spp.*

There are 12 *Vibrio* species that are regarded as foodborne pathogens, which include *Vibrio cholera, V. parahaemolyticus* and *V. vulnificus*. *Vibrio cholera*, the causative agent of cholera is one of the few with epidemic and pandemic potential. The seventh pandemic of cholera, in 1961 involved more than 100 countries affecting more than 3 million persons and causing death of many thousands. It is a part of free living bacterial flora in natural waters and is the source of contamination for fresh horticulture produce. Water and shell-fish are the most common modes of transmission. The symptoms of the disease include the potentially explosive fatal dehydrating diarrhoea, abdominal cramps along with nausea, vomiting and fever (Oliver and Kaper, 2002).

2.1.12 *Yersinia enterocolitica*

The genus *Yersinia* comprises 11 species within family Enterobacteriaceae. *Yersinia enterocolitica* was first reported in 1930s and is a Gram-negative, rod-shaped and facultative anaerobe. It is a versatile pathogen with ability to adapt widely within and outside the mammalian hosts. It has gained significance due to its nature of causing serious post-infectious complications. Yersiniosis leads to acute intestinal infections such as mesenteric lymphadenitis, ileitis, which give rise to symptoms of appendicitis and post-infectious manifestations like arthritis. The ability of the organism to grow in refrigerated conditions makes it a pathogen of concern. Pigs and food products of porcine origin are the major sources of human infection. The organism is prevalent in many countries, but found frequently in those nations with temperate climate (Kapperud, 2002).

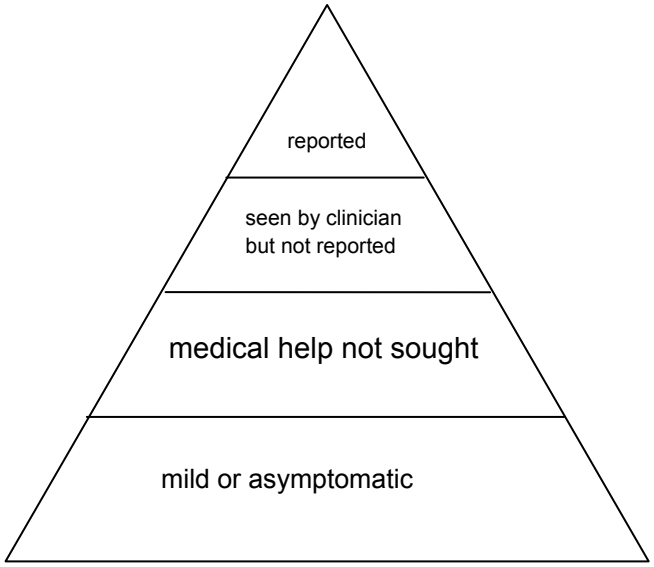
2.2 FUTURE OF FOODBORNE DISEASES FROM A SOCIETAL PERSPECTIVE

As population increases, the need for food safety is likely to increase. The steady increase in the need for space for production of safe food and at the same time need to have land space for waste disposal results in ecological pressure, which may result in the emergence and dominance of new pathogens. This has necessitated scientific and technological intervention for mass production of safe foods. Besides, the increased travel of the public has enhanced the risks that get associated with exposure to pathogens. The other areas affecting the future of foodborne disease are public health support programs, sanitary disposition of waste water and agri-based matter. This may lead to the collaborations among the agricultural, food, regulatory, research and public health communities. As food and water are essential to life, efforts to ensure their safety continue to be the thrust area to society (Snowdon et al. 2002).

The following is the table illustrating the epidemiology of major foodborne pathogens

Country, Period & (Reference)	*Salmonella* spp	*Staphylococcus aureus*	*Clostridium perfringens*	*Campylobacter* spp.	*Bacillus cereus*	*E. coli*	*Listeria monocyto-genes*
Canada 1993 (www.hc-sc.gc.ca)	39	6	10	7	8	14	2
Denmark 1985-89 (www.who.int)	5.2	2.4	5.8	0.4	2	0.2	0.4
England 1986-89 (www.who.int)	438.3	10	53	53.3	19.3	0	0.3
France, 1997	201	32	13	0	1	0	0
Germany 1992	177	1	6	0	2	1	1
Japan 1987-90	110.8	120.3	19	31.8	10.3	20	0
Netherlands 1985-89	8	1.6	2.4	2	4.8	0	0
Spain 1994	379	39	13	2	1	9	0
Sweden 1990-92	10.3	2.7	3.7	0.7	0.7	0	0
USA 1992	81	6	12	6	3	3	0

Most of the cases of foodborne illness go unreported because of the common symptoms like diarrhoea, vomiting, nausea, abdominal cramps and fever which resemble almost common influenza. Owing to the mild symptoms and self-limiting nature of the illness, the victims often do not seek immediate medical intervention. Added to this, the Medical Practioners may fail to report the numbers to Central Health Agencies. Hence, the number of cases recorded is just the 'tip of the iceberg' with regard to the actual numbers. The different degrees of foodborne illness are shown schematically as a pyramid.



2.3 HISTORY AND PUBLIC HEALTH SIGNIFICANCE OF *B. CEREUS*

Bacillus cereus is a common soil saprophyte and can easily spread to many types of foods including vegetables, dairy and meat products. This is possible, because of the heat resistant nature of its endospores. It is also isolated from air, water, animal and plant materials. It was originally isolated from air in a cow shed by Frankland and Frankland in 1887. *Bacillus cereus* is known to cause two different types of diseases namely the diarrhoeal type and emetic type. As early as 1906, an outbreak of foodborne illness was caused by a spore forming bacillus that Lubenau called it as *Bacillus*

peptonificans. The characteristic of the organism closely resembled those of *B. cereus.* But, the first diarrhoeal type was recognized after a hospital outbreak associated with contaminated vanilla sauce in Oslo, Norway in 1948. After the recognition of first outbreak, Steinar Hauge isolated the causative agent, grew it to 6 $\log_{10}$ CFU/ml and drank 200 ml of the culture. He developed abdominal pain and diarrhoea after 13 h which lasted for 8 h. Since then, *B. cereus* has been considered as a causative agent of diarrhoea (Hauge, 1955).

After nearly 20 years with several outbreaks being reported, the emetic type was documented in London and the implicated food was fried rice. The emesis resembles staphylococcal type of gastrointestinal disease. The illness was characterized by nausea and vomiting as symptoms in victims after 1-6 h of consumption of fried rice from a Chinese restaurant (Mortimer and McCann, 1974). The diarrhoeal type is caused by enterotoxin produced by growth of vegetative cells in the small intestine, whereas the emetic toxin is preformed in food (Granum, 1994).

Bacillus cereus food poisoning usually occurs as a result of spores surviving cooking or pasteurization, followed by germination and multiplication when food is not adequately refrigerated. The illness caused by *B. cereus* is under-reported, because both types of manifestations are mild in nature, self-limiting, last less than 24 h and often does not need medical intervention. Nevertheless, extreme forms of the diarrhoeal type of *B. cereus* have been reported which include rare fatalities. Apart from causing gastro-intestinal disorders, it occurs as a virulent ocular pathogen and is known to cause conjunctivitis, panophthalmitis, keratitis, iridocyclitis and orbital abscess. It can also act as opportunistic pathogen causing respiratory tract and wound infections (Kramer and Gilbert, 1989).

Bacillus cereus is morphologically, culturally, biochemically and genetically closely related to *B. thuringiensis* and the laboratory procedures routinely practiced do

not clearly distinguish between the two species of *Bacillus*. *Bacillus thuringiensis* used as a biocontrol agent against insects is known to produce enterotoxin and also involved in food poisoning outbreaks (Drobniewski, 1993; Jackson et al. 1995). This has added to the ambiguity of the actual number of health hazards resulting from *B. cereus* along with the under reporting of the cases as mentioned earlier.

Bacillus cereus is known to spread across through cross-contamination of the foods involved. It can occur in a wide variety of foods including cereals, especially rice (Shah et al. 1996; Finlay et al. 2002), milk and milk products (Reyes et al. 2007; Lin et al. 1998), legume-based foods (Roy et al. 2007), ready-to-eat foods (Harmon and Kautter, 1991; Varadaraj et al. 1992; Rosenquist et al. 2005), meat and poultry products (Smith et al. 2004; Guven et al. 2006) and spices (Seenappa and Kempton, 1981).

2.4 INCIDENCE AND OUTBREAKS OF *B. CEREUS* FOOD POISONING

The incidence of *B. cereus* is less reported in view of the transient and mild nature of the symptoms. The incidence and nature of foodborne illness vary from one country to another. It was a prominent pathogen found in 3% of the food samples taken from hot meals served in passenger flight carrier during 1991-1994 (Hatakka, 1998). In Japan, the emetic type is significantly prevalent and is reported 10 times more than the diarrhoeal type (Shinagawa, 1993), whereas in Europe and North America the diarrhoeal type is reported predominantly. In Japan, North America and Europe, the percentage of outbreaks due to *B*. *cereus* vary from 1 to 47% and cases range from about 0.7 to 33%. It is approximated that there are about 84000 cases of foodborne illness caused by *B. cereus* annually in the United States at a cost of US $43/- per person and a total cost of US $36 million. Iceland, Netherlands, Canada, England, Scotland, Wales and Norway also reported high number of such cases (Kramer and Gilbert, 1989).

In India, sporadic incidences of *B. cereus* food poisoning have been reported. Various studies report the incidence of the organism in foods like milk, milk products, ice-creams, rice, spices, vegetables and pulses (Kamat et al. 1989; Warke et al. 2000; Bedi et al. 2005). India being a subtropical country with diverse foods, there are ample chances for a majority of the incidences to have gone unnoticed or unreported.

Bacillus cereus can contaminate wide variety of foods and can survive extended storage in dried food products. The trend towards use of refrigerated processed foods with extended durability and the increasing number of elderly and immuno-compromised people does raise the importance of *B. cereus* as etiological agent of foodborne illness. Due to the heat and acid resistance, it is difficult to eliminate low numbers of spores from foods by pasteurization and routine sanitation. Preventing the germination of spores and containing the growth of vegetative cells could be one of the effective strategies to control the incidence of this organism.

2.5 TAXONOMY AND CHARACTERISTICS OF *B. CEREUS*

Bacillus cereus is a Gram-positive, endospore-forming, motile, facultative anaerobic and rod-shaped bacterium belonging to family Bacillaceae. The genus *Bacillus*, described in Bergey's Manual of Systematic Bacteriology is too diverse and significant changes have been proposed for its classification (Claus and Berkeley, 1986). Initially *Bacillus* species were classified based on only two characters namely aerobic growth and endospore forming nature. However, this resulted in ambiguity, wherein other organisms with unrelated physiology, habitats, ecology and genetic make-up gained place and the level of heterogeneity increased thus making the classification difficult (www.textbookofbacteriology.net).

2.5.1 Taxonomic status

In the first edition of Bergey's Manual of Systematic Bacteriology (Holt, 1984), the G+C content of known species of *Bacillus* ranged from 32 to 69%. This, in addition to DNA hybridization tests indicated the level of heterogeneity of the genus. The G+C content varied not only between the species, but also among different strains. The profound differences in G+C content observed sometimes, made it difficult to categorize the *Bacillus* species. This was overcome in the second edition of Bergey's Manual of Systematic Bacteriology (Garrity et al. 2003), wherein, the phylogenetic classification pattern brought the genus *Bacillus* in class Bacillales. Bacilli includes the Order Bacillales and the Family Bacillaceae. At present, there are 37 new genera in this family on the level with *Bacillus.* An analysis of 16S rRNA sequences from *Bacillus* members showed that there are five genera or rRNA groups within the genus *Bacillus* (Ash et al. 1991a). Many new species were identified and added subsequently, taking the number of genera to around 16.

The status of *B. cereus* in the taxonomic hierarchy as illustrated in the second edition of Bergey's Manual of Systematic Bacteriology (Garrity et al. 2003) is as follows:

Class: Bacilli

Order: Bacillales

Family: Bacillaceae

Genus: *Bacillus*

Species: *cereus*

The new methods of analysis have classified the different species into a variety of bacterial taxa and have been continuously modified in a dynamic fashion. As a result, *B. cereus* group comprises six very closely related species: *B. cereus, B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides* and *B. weihenstephanensis*. Earlier, these species were considered as distinct from each other, because of their phenotypical

differences. Later studies with molecular biology approaches showed that they have highly identical 16S and 23S rRNA sequences, indicating that they have diverged from a common evolutionary line and be considered as belonging to the same species (Ash and Collins, 1992).

In this context, strains of *B. anthracis* have been related to the other species within the *B. cereus* group based on rRNA sequences. However, it is the most distinctive member of the group taxonomically and due to its ability of capsule formation and virulent pathogenicity by production of toxins that causes carbuncles in animals and humans, leading to anthrax disease. Similarly, comprehensive studies and genomic analysis of *B. cereus* and *B. thuringiensis* indicate that there is no taxonomic basis for separate species status. Nevertheless, the name *B. thuringiensis* is retained for those strains that synthesize a crystalline inclusion (cry protein) or delta-endotoxin that is entomicidal to larvae of specific insects, allowing the use of bacterial products as biocontrol agent. Also, the *cry* genes are usually located on plasmids and their loss makes the organism indistinguishable from *B. cereus* (Carlson et al. 1996).

Despite these phenotypic differences, comparison of their 16S rRNA nucleotide sequences showed less than 1% variation amongst the species studied (Ash et al. 1991b), but a cut-off of 3% divergence is recommended as a conservative criterion for the demarcation of species, thus advocating the concept of single species. Hence, the analysis of the sequences of these genes might be used to characterize a strain as belonging to the *B. cereus* group without being able to identify the species. A comparative analysis of the genomes of *B. cereus, B. anthracis* and *B. thuringiensis* shows that the chromosomes of these species are highly similar thus indicating a conserved gene order. In addition to this, extensive studies on strains of *B. cereus, B. thuringiensis* and *B. anthracis* using advanced techniques like multi-locus enzyme electrophoresis (Helgason et al. 2004), sequencing of discrete protein-coding genes

(Helgason et al. 2000), amplified fragment length polymorphism fingerprinting (Ticknor et al. 2001) and multi-locus sequence typing (Helgason et al. 2004) have proposed that *B. thuringiensis* and *B. anthracis* should be classified as sub-species of *B. cereus.*

Thus, currently there is no consensus as to whether these bacterial species should be considered as separate species or variants of different species. A few of the recent studies have provided sufficient genetic differentiation among *B. cereus, B. thuringiensis* and *B. anthracis* (Radnedge et al. 2003) and are regarded as separate species for all practical purposes. The availability of complete genome and plasmid sequences of many strains of the *B. cereus* group allows for extraordinary advances of the knowledge of their chromosomal structure, gene content and pathogenic phenotypes. Efforts in this direction, to elucidate the origin of the differences among the members of this group of bacteria are needed.

2.5.2 Characteristics

The colonies of *B. cereus* on the agar medium have a dull or frosted appearance with irregular and spreading nature. The individual cells are typically large of 1-3 µm in length and 1-1.2 µm in width. Motility is with the help of peritrichous flagella, often occurring in chains. The mol% G+C of the DNA is approximately 32-38. The spores formed are central to terminal, ellipsoid or cylindrical that does not extend sporangia. The organism sporulates easily on most media after 2 to 3 d and loses motility during early stages of sporulation. L-alanine, glycine or a neutral amino acid and purine ribosides are known to induce germination. The Decimal Reduction Time (*D*-value) varies approximately from 22 to 30 min at 95°C by moist heat. The spores are also resistant to irradiation and dessicants. Their hydrophobic nature and the presence of appendages on their surfaces, enable them to adhere to several types of surfaces. Their adhesiveness makes difficult the physical removal from the surfaces during cleaning and sanitation.

The temperature range for the growth is generally between 5 and 50°C. Some strains can grow slowly at 10% sodium chloride concentrations. The minimum water activity for growth is between 0.91 and 0.93 and it can grow over a pH range of approximately 4.4 to 9.3. However, these limits for growth are not absolute and are dependent on several factors including the genetic make-up of the strain. It has a requirement for amino acids as growth factors, but vitamins are not very essential (Griffiths and Schraft, 2002). The details of morphological, cultural and biochemical characteristics of *B*. *cereus* have been very well documented in the literature (Garrity et al. 2003).

2.5.2.1 Typing of B. cereus

2.5.2.1.1 Serotyping

Serotyping of *B. cereus* is based on the flagellar (H) antigens. It is reported that spore antigen is species specific and the H antigen is strain specific (Norris and Wolf, 1961). A typing pattern of use in epidemiological investigations of food poisoning outbreaks was developed (Taylor and Gilbert, 1975; Gilbert and Parry, 1977). The scheme employs a basic set of more than 20 agglutinating sera and a constant monitoring for updating and expansion of the set is needed. Some serotyping has been associated with both emetic and diarrhoeal type of illnesses. Multiple serovars were also recovered from foods involved in illness. Bedi et al. (2005) reported the incidence of serotypes H-1, H-8, H-17, H-19, H-20, H-25 and some untypable strains from milk and milk products.

2.5.2.1.2 Biotyping

Biotyping or biochemical differentiation between the strains causing the emetic and diarrhoeal illnesses has been a subject of much interest. Strains causing the emetic illness are unable to hydrolyse starch (Shinagawa et al. 1985) or ferment Salicin (Gilbert

and Taylor, 1976), although slow fermentation of salicin has been reported in some cases (Gilbert et al. 1981). Using Analytical Profile Index (API), it was shown that the differentiation of emetic strains and diarrhoeal strains was possible based on the inability to hydrolyze dextrin, starch and glycogen (Logan and Berkeley, 1981). However, some workers differ in their opinion due to the inconsistent differences between diarrhoeal and emetic strains (Major et al. 1979).

2.5.2.2 *Plasmid profiling and antimicrobial resistance of B. cereus*

Plasmids isolated from *B. cereus* strains have been in the range of molecular weight from 1.6 x 10^6 to 105 X 10^6 daltons. Bacteriocin production was observed and attributed to a 45 X 10^6 dalton plasmid (pBC7) from *B. cereus* DSM 336 and tetracycline resistance to a 2.8 X 10^6 plasmid from *B. cereus* GP7 (Bernhard et al.1978). Bacteria resistant to several antimicrobial drugs gain entry into human population mainly through foods of animal origin by virtue of the animal feeds being contaminated with drug resistant strains. Such strains act as potential sources of food related public health hazards and difficult to eliminate by narrow range antibiotics. The plasmids, which are extra-chromosomal DNA elements of small size, are known to play a leading role in conferring antimicrobial resistance. It was reported that strains of *B. cereus* from different dairy and meat products were susceptible to ampicillin, cephalothin, oxacillin and streptomycin (Schlegelova et al. 2003). Hassan and Nabbut (1996) reported that diarrhoeal strains of *B. cereus* obtained from rice, dehydrated milk and soil were resistant to amoxicillin, oxacillin and pencillin, while being susceptible to gentamycin and vancomycin. Susceptibility to clindamycin and erythromycin was found to be inconsistent among the isolates.

Similarly enterotoxigenic strains of *B. cereus* isolated from different cooked foods like rice, noodles, dairy products (fermented milk, soft ice cream, pasteurized milk,

pasteurized fruit or nut flavoured milk, reconstituted milks and milk powders) were found to be resistant for carbenicillin, nalidixic acid, ampiciilin, cloxallin, tetracycline and susceptible to chloramphenicol, erythromycin, gentamycin and streptomycin (Wong et al. 1988; Rusul and Yaacob, 1995). Studies on the isolates of *B. cereus* obtained from variety of foods in India showed high resistance of the strains to ampicillin, trimethoprim, colistin, rifampicin and nitrofurantoin (Shah et al. 1996). Strains of *B. cereus* collected from Danish pig farms were found resistant to bacitracin, erythromycin, penicillin and streptomycin. Variations in the resistance levels were observed when the soil before and after spread of animal waste were compared (Jensen et al. 2001). Ombui et al. (1996) studied the plasmid profile and antimicrobial resistance of several isolates of *B. cereus* and found all resistant to ampicillin with a high rate of resistance to cotrimoxazole and susceptible to streptomycin. No apparent relationship between the presence of plasmids and drug resistance was observed.

2.5.2.3 Pathogenic/Virulence/Toxigenic traits associated with B. cereus

There are two types foodborne illness caused by *B. cereus*. The first type caused by enterotoxin(s), results in diarrhoea and the second type induces nausea and vomiting. In a few number of cases, both types of symptoms are witnessed, probably due to the production of both types toxin. There has been some difference of opinion about whether enterotoxin(s) are preformed in foods and cause intoxication. A review into the literature shows that the incubation time (>6 h; average 12 h) is likely too long for diarrhoeal illness to be caused by preformed enterotoxin (Kramer and Gilbert, 1989). Also animal model experiments showed that enterotoxin(s) is degraded as it proceeds towards the ileum. The enterotoxin can be also be preformed in foods, but the number of *B. cereus* cells in the food should be at least two orders of magnitude more than that necessary to cause food poisoning . Generally, the products with such large number of cell population

of *B. cereus* are no longer acceptable to the consumer, although food containing >10^7 cells /ml may not always appear spoiled (Granum, 1994).

The general comparison of characteristics of the two types of foodborne illness is as follows:

Characteristic	Diarrhoeal syndrome	Emetic syndrome
Infective dose	10^5-10^7 CFU/g or ml (cells ingested)	10^5-10^8 CFU/g or ml
Toxin produced	In small intestine of host	Preformed in foods
Type of toxin	Protein; enterotoxin(s)	Cyclic peptide
Incubation period (h)	8-16 (occasionally >24)	0.5-5
Duration of illness (h)	12-24 (occasionally several days)	6-24
Symptoms	Abdominal pain, watery diarrhoea, occasionally nausea	Nausea, vomiting, malaise
Foods implicated	Meat products, soups, vegetables, Puddings and sauces, milk and Milk products	Fried and cooked rice, pasta, noodles

Source: Shinagawa, 1993; Granum, 1994; Kramer and Gilbert, 1989

2.5.2.3.1 Diarrhoeal enterotoxin

Stiener Hauge was the first to provide comprehensive description of *B. cereus* diarrhoeal syndrome in the 1950's after investigating four Norwegian hospital outbreaks. A high count of the organism was recovered from the vanilla sauce implicated as the vehicle of outbreak. To establish the toxicity of the suspected organism, he inoculated high number of cells in sterile vanilla sauce, incubated it for 24 h at room temperature and consumed 200 ml of the sauce. He developed abdominal pain, diarrhoea and rectal nesmus within 13 h and the symptoms lasted for 8 h (Hauge, 1955).

The general properties of diarrhoeal enetrotoxin(s) are as follows:

Trait	Property or Activity
Structure	Three component protein complex; more than one toxin involved
Heat stability	Destroyed at 55°C after 20 minutes
pH stability	Unstable < 4 and > 11
Effect of proteolytic enzymes	Inactivated by pronase and kinase
Assay procedures	Immunoassays, rabbit ileal loop, cytotoxic assay
Mode of action	produced in intestine, reverses ion inlflux and fluid accumulation

Reference: Griffiths and Schraft, 2002

While the general symptoms include watery diarrhoea and abdominal cramps, occasionally nausea and vomiting are also experienced. The symptom profile resembles *Clostridium perfringens* food poisoning. The onset time of the diarrhoeal syndrome generally ranges from 8 to 16 h and the symptoms resolve in 12 to 14 h. Outbreaks with unusual onset and duration have also been reported.

The foods associated with the syndrome include meats, fish, vegetables, soups, sauces and dairy products. A large number of cases do involve protienaceous dishes. The foods implicated in the illness had a concentration of cells ranging from 10^4 to 10^{11} cells/g, thus indicating a high number of cells are needed to cause disease, (Kramer and Gilbert, 1989; Granum and Lund, 1997).

The detection of diarrhoeal enterotoxin is undertaken by different methods. These include ileal loop, vascular permeability and monkey feeding assays. Even today, rabbit ileal loop assay is considered as an ideal *in vivo* method for the detection of enterotoxigenic activity (Bergdoll, 1988). Also, cytotoxicity in cell culture assays has been used as an indication of the presence of *B. cereus* diarrhoeal toxin in crude

samples like food extracts and culture supernatants (Beattie and Williams, 1999). The method has a limitation in that although the enterotoxin is considered cytotoxic, a variety of enzymes and toxins produced by *B. cereus* can act on cell membranes and therefore also produce a cytotoxic effect.

Two commercial kits to detect *B. cereus* enterotoxin were independently developed. Oxoid developed a *B*. *cereus* enterotoxin reverse passive latex agglutination kit (BCET-RPLA kit, Denka Seiken Ltd., Tokyo, Japan), which is a semi-quantitative assay. The kit was found to target and detect the L_2 component of the tripartite toxin HBL. Another kit, *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (Tecra Bioenterprises, Pvt. Ltd., Roseville, Australia) allows detection of toxin using a double-sandwich enzyme immunoassay. The antigen detected by the kit is a 45 kDa protein, which was later identified as NheA from the Nhe complex (Beecher and Wong, 1994a). Both the kits have been used widely (Christiansson, 1993; Day et al. 1994) and the results were compared with each other and with those of cytotoxicity tests in tissue culture (Buchanan and Schultz, 1994; Rusul and Yaacob, 1995). The kits had some discrepancies in them as encountered during their application studies, which was because each kit detects a different eneterotoxin component. The Oxoid kit detects L_2 from HBL and the Tecra kit detects NheA from Nhe complex. This may of help as indicators of which samples contain a specific toxin complex. However, they have limitations of use to confirm the presence of biologically active toxin, because both complexes require all the three components for biological activity.

The different diarrhoeal enterotoxins produced by *B. cereus* are briefly described in the following paragraphs

1. Haemolysin BL (HBL)

The toxin was first isolated by Beecher and Macmillan (1990). It is a tripartite toxin produced by *B. cereus*, from a strain F837/76. This is the only factor that has been highly

purified (Beecher and Wong, 1994b) and established to be a diarrhoeal toxin by the ligated rabbit ileal loop assay (Beecher et al 1995a). The three components of HBL namely B, L_1, and L_2, purified from strain F837/76 have molecular masses of 37.8, 38.5 and 43.2 kDa respectively, and pI values of approximately 5.3 (Beecher and Wong, 1994c). The genes *hblC* (L_2), *hblD* (L_1), and *hblA* (B) are arranged in tandem in an operon (Ryan et al. 1997), with the promoter located upstream of *hblC*. Alignment of the deduced amino acids sequences of the three components revealed significant homology. The proteins are 20 to 24% identical to each other. Structural analysis of the HBL proteins indicates that all the three components consist of almost entirely of alpha-helix. Components of B and L_1 contain predicted transmembrane segments of 17 and 60 amino acids residues, respectively, in the same position, whereas L_2 does not contain predicted transmembrane segments. These similarities indicate that the HBL components resulted from the duplication of a common gene (Beecher, 2001).

All the three components in HBL are required for the biological activity. HBL produces a characteristic discontinuous haemolysis pattern on blood agar (Beecher and Wong, 1997). Hemolysis begins several millimeters from the edge of a colony or a well containing HBL, forming a ring-shaped clearing zone (discontinuous). Gradually, the zone moves inward towards the source. The potency of the enterotoxin varies depending on the species of the mammalian blood tested. Beecher and Wong (1997) found that the discontinuous haemolysis pattern is mediated by the B and L_1 components. Sheep erythrocytes do not lyse when incubated with the B component alone. Interestingly, the erythrocytes become sensitized or primed and are rapidly lysed with the addition of L_1 and L_2. Excess concentrations of B or L_2 interfere and inhibit the activity of each other. The L_2 component is needed for lysis, but does not interfere with the action of L_1 or B. Hence the haemolysis of erythrocytes in the blood agar plate assay occurs at the point in the diffusion gradient (away from the well), wherein appropriate

concentrations of both B and L_1 exists. A high degree of molecular heterogeneity exists in HBL from different strains. Individual strains produce one or two various combinations of single or multiple bands of each component. Some strains are known to produce only one or two of the three HBL components (Schoeni and Wong, 1999; Radhika et al. 2002).

In addition to its haemolytic activity, HBL is dermonecrotic, increases vascular permeability and is cytotoxic to Chinese hamster ovary cells and retinal tissue both *in vitro* and *in vivo* (Beecher et al. 1995a). The toxin causes fluid accumulation in the rabbit ileal loop assay and necrosis of villi, sub-mucosal edema and interstitial lymphocytic infiltration (Beecher et al. 1995). HBL is shown to induce pores in eukaryotic cell membranes, with each of the components binding the membrane independently and reversibly (Beecher and Wong, 1997). A hypothesis proposed for this was, once bound, the components oligomerize and form transmembrane pores consisting of at least one of each component. The transmembrane segments in B and L_1 may serve as mediators of oligomerization (Beecher, 2001).

2. Non-haemolytic enterotoxin (Nhe)

Granum et al. (1996) after screening over 300 *B. cereus* strains concluded that there was at least one enterotoxin in addition to HBL, implicated in the enterotoxin induced illness. The strain of *B. cereus* 0075-95 responsible for a Norwegian diarrhoeal outbreak was used to characterize Nhe (Lund and Granum, 1996). Later, Granum et al. (1999) cloned and sequenced *nhe* from *B. cereus* strain 1230-88 (EMBL accession no. Y19005). The *nhe* operon comprises three open reading frames that correspond to the genes *nheA, nheB* and *nheC.* The deduced sizes of the encoded proteins were 41 kDa, 39.8 kDa and 36.5 kDa and predicted isoelectric point values (pI) of 5.13, 5.61 and 5.28, respectively. The proteins of Nhe components show homology with each other and also

to a considerable extent with HBL components. NheB and NheC have two and one predicted transmembrane helices, respectively, located in the same position. Similarities were also observed in predicted transmembrane helices for the proteins. This shows that HBL and Nhe are homologs in a family of tripartite toxins (Beecher, 2001).

Studies into genome mapping of *B. cereus* have shown that the sizes of the chromosome vary to a large extent, ranging from 2.4 to 6.3 Mb and extra-chromosomal bands in the form of plasmids are often observed (Kolsto et al. 1990; Carlson et al. 1992). The genetic organization of one region of the chromosome appears to be constant and the other variable in terms of presence and location of genes. The constant region comprises house-keeping and ribosomal genes, whereas the genes in the variable region are often coded by plasmids (Carlson et al. 1992). The *hbl* operon is located in the variable region, and the *nhe* operon is in the constant portion of the *B. cereus* chromosome (Carlson et al. 1996). The production of both HBL and Nhe is being widespread (Rusul and Yacoob, 1995), some strains produce only one or two of the HBL components and others possess the HBL genes, but the proteins could not be expressed (Schoeni and Wong, 1999). While HBL is produced by *B. mycoides*, both HBL and Nhe are produced at maximum level in their exponential phase by *B. cereus* and *B. thuringiensis* (Prüß et al. 1999).

PlcR is shown to be a pleitropic regulator that controls the expression of at least 15 genes, many of which encode for virulence factors in *B. cereus and B. thuringiensis*. These genes are widely spread, do not form pathogenicity island and encode for degradative enzymes, cell-surface proteins and toxins that include HBL and Nhe (Agaisse et al. 1999). A highly conserved palindromic region ($TATGNAN_4TNCATA$), known as the PlcR box, is the specific recognition site for PlcR activation and is located at different positions upstream of the transcription start site of the target gene (Okstad et al. 1999).

3. Enterotoxin T (BceT)

The toxin is a single component protein with a molecular weight of 41 kDa. It was absent in 57 of the 95 strains of *B. cereus* and in 5 of 7 strains involved in foodborne illness. The toxin lacks a signal sequence and is released only after *B*. *cereus* cell lysis (Granum et al. 1996).

4. Cytotoxin K (CytK)

This is a single component hemolytic protein of 34 kDa size and resembles the beta-toxin of *Cl. perfringens* (and other related toxins). It was implicated in a necrotic enteritis outbreak in a nursing home in France in 1998, wherein several encountered diarrhoea and three died (Lund et al. 2000).

5. Cereolysin

Cereolysin is a thiol activated protein and cross reacts with streptolysin-O. It is heat labile and slightly susceptible to proteolysis. It is lethal when injected into mice and is inhibited by cholesterol and serum (Cowell et al. 1976).

6. Phospholipase C

Phosphatidylinositol hydrolase (PIH) is a protein that specifically hydrolyses phosphatidylinositol (PI) and PI-glycan-containing membrane anchors, which are major structural components of membrane proteins. The enzyme has 329 amino acids with a signal peptide of 31 amino acids and a molecular weight of 34 kDa (Kuppe et al. 1989). Phosphatidylcholine hydrolase (PCH) hydrolyses phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine (Little et al. 1975). It is synthesized as a 283 amino acid protein with 245 amino acids in the mature protein and size of 27 kDa.

7. Sphingomyelinase

Sphingomyelinase is heat stable metallo-enzyme (Mg^{2+}) with a size of 34 kDa and hydrolyses sphingomyelin (Ikezawa et al. 1986). It is active through hot-cold incubation (37 to 4°C). It is synthesized as a 333 amino acids and has 306 amino acids in mature protein (Gilmore et al. 1989).

2.5.2.3.2 Emetic toxin syndrome - Cerulide toxin

After 20 years of diarrhoeal syndrome being established as disease, emetic syndrome was recognized as the cause for emesis leading to nausea and vomiting. Several emetic incidences in U.K. during early 1970‘s were associated with fried rice. The illness was characacterized by nausea and vomiting in a period of 1-6 h after consuming rice (Mortimer and McCann, 1974). The emetic syndrome is more associated with farinaceous foods like rice, pasta and noodles (Kramer and Gilbert, 1989). The incidence is more, if boiled rice is kept at ambient temperature for long time and then quickly fried before serving. The emetic strains were isolated invariably from sources like infant formula, skim milk powders and other foods (Holmes et al. 1981).

Characteristics

The emetic toxin is a heat stable, single peptide also called cereulide which is preformed in foods and resembles staphylococcal enterotoxin. It consists of a ring structure of three repeats of four amino acids and/or oxy acids: [D-*O*-Leucine –D-Alanine-L-*O*-Valine-L-Valine]$_3$. The ring structure (dodecadepsipeptide) has a molecular mass of 1.2 kDa and is closely related chemically to the potassium ionophore like valinomycin (Agata et al. 1994). The toxin is resistant to heat, pH, proteolysis, toxic at low dose and is not antigenic (Kramer and Gilbert, 1989). It is hydrophobic and not easily solubilized in

aqueous solutions and delivered to the target cells by getting bound or dissolved in carriers found in foods (Turnbull et al. 1979).

The toxin is known to stimulate the vagus afferent through binding to the serotonin *5*-HT_3 receptor. It also causes swelling of mitochondria by acting as potassium ionophore like valinomycin (Agata et al. 1995b). It can cause cellular damage in animal models and lead to fulminate liver failure (Mahler et al. 1997) and has also been reported that cereulide inhibits human natural killer cells and might therefore have an immunomodulating effect (Paananen et al. 2002).

A relatively high amount of the emetic toxin is known to be produced during the beginning of stationary phase of the growth and also depends on the incubation temperature and the culture medium (Finlay et al. 2000). The presence of glucose is known to enhance cereulide production, while excessive amounts of leucine, isoleucine and glutamic acid suppress the production (Agata et al. 1999). In food systems the highest rate of toxin production was observed in aerated milk, followed by rice and pasta and low in bread and cakes (Agata et al. 2002). The ability to elaborate cereulide toxin varies among different emetic strains (Haggblom et al. 2002). Nevertheless, the emetic strains are believed to have a same genetic linkage (Pirttijarvi et al. 1999). Altayar and Sutherland (2006) reported that although *B. cereus* is a common isolate, incidence of the emetic strain is rare.

Molecular basis for cereulide production

The emetic toxin, cereulide is known to be a putative product of non-ribosomal peptide synthetase (Ehling-Schulz et al. 2005; Toh et al. 2004).The chemical structure of cereulide leads to a prediction, that it is enzymatically synthesized by a non-ribosomal peptide synthetase (NRPS) with alternating peptide and ester bonds, D-amino acids and a cyclic structure. NRPSs are large multifunctional proteins that have a modular

organization. One module contains all catalysts which are needed for incorporation of one amino acid residue into the product. Highly conserved motifs are present within these modules that can be used for a universal PCR approach to identify unknown parts of NRPS. Sequence analysis of one of the amplicons of the conserved sequences showed a DNA fragment that was predicted to encode a valine activation NRPS module. Since valine is one of the four monomers of cereulide, the gene fragment identified was suspected to belong to the genetic locus responsible for cereulide synthesis (Turgay and Marahiel, 1994).

The different characteristic features of cerulide are shown below

Properties of the emetic toxin cerulide

Trait	**Property or Activity**
Molecular mass	1.2 kDa
Structure	Ring-shaped peptide
Isoelectric point	Uncharged
Antigenic	No
Biological activity in primates	Vomitting
Receptor	5-HT_3 (stimulation of the vagus afferent)
Ileal loop tests (rabbit, mouse)	None
Cytotoxic	No
HE_p-2 cells	Vacuolation activity
Stability to heat	90 min at 121°C
Stability to pH	Stable at pH 2-11
Effect of proteolysis (trypsin, pepsin)	None
Conditions under which toxin is produced	In food: rice and milk at 25-32°C
Mechanisms of toxin production	Presumed to be enzymatically synthesized

Source: Kramer and Gilbert, 1989; Shinagawa, 1993; Agata et al. 1994; Agata et al. 1995b

2.6 ISOLATION AND DETECTION PROTOCOLS FOR *B. CEREUS* FROM FOODS

2.6.1 Conventional methods

Consumer awareness with respect to food safety and increase in the demand for microbiologically safe foods has been playing a prominent role in detection and enumeration of foods. In this background, the reliability of the techniques for detection and enumeration of foodborne pathogens has taken a centre-stage. It is expected that the method employed should be (1) simple and rapid, (2) accurate and reproducible and (3) economically feasible. *Bacillus cereus* being ubiquitous, the surveillance protocols must be targeted at monitoring and controlling the ingredients used for the preparation of food, the finished product and the environmental process. These aspects are considered in the choice of the isolation media, requisite selective and inhibitory agent(s) to enhance isolation and recovery of specific organism of interest and at the same time suppress the background microflora. An ideal and effective *B. cereus* isolation medium should suppress competitive microflora so that easy and reliable detection of typical strains of *B. cereus* is possible.

2.6.1.1 Culture media

The quantitative estimation of *B. cereus* in foods is performed by a standard plate counting method. However, a most probable number (MPN) technique using Trpticase soy polymyxin B (100 IU/ml) is used for estimating the organism in (i) foods expected to contain <10 CFU/g or (ii) dehydrated starch foods for which the plate count is inappropriate (Lancette and Harmon, 1980). Hauge (1955) practiced direct surface plating of *B. cereus* on Blood Agar (BA) while Gilbert and Taylor (1976), advocated the use of horse blood agar for the enumeration. The approach is based on the characteristic colony appearance. A method of surface spreading with Polymyxin B solution on Columbia base horse blood agar plates was also practiced (Kramer et al.

1982). Citrate egg yolk lithium chloride polymyxin B (CELP) agar was the first selective agar developed for the enumeration of endospores and vegetative cells from milk and dairy products with Polymyxin B and lithium chloride as selective agents. The egg yolk reaction of McClung et al. (1946) was made use of for the presumptive identification with citrate added to enhace the effect (Donovan, 1958).

Later in mannitol egg yolk polymyxin B (MEYP) agar, the single egg yolk diagnostic system was extended to double by supplementation of mannitol/phenol red combination with an increased concentration of (100 IU/ml) of Polymyxin B (Mossel et al. 1967). The formulation was improved by the use of fluorescein-labelled anti-exosporium antibody technique as a rapid and less cumbersome serological confirmation (Kim and Goepfert, 1971a). This needed a culture medium that supported free endospore production within 24 h, for which MEYP was found to be nutrient rich. A new basal medium (KG) was formulated which contained low levels of peptone (0.1% w/v), no carbohydrate, Polymyxin B as selective agent, egg yolk emulsion for differentiation and Phenol red (0.0025%) to facilitate the observation of egg yolk precipitation (Kim and Goepfert, 1971b). Kendall's mannitol egg yolk bromocresol purple agar (BCM) was the first nutrient limited agar medium which was developed primarily to assist with differentiation of *B. cereus* from other *Bacillus* species (Gilbert and Taylor, 1976).

In MEYP and KG media, there was a poor differentiation of mannitol utilizing organisms after incubation and egg yolk reactions were weak. These drawbacks led to the formulation of new *B. cereus* selective medium namely polymyxin B pyrvate egg yolk mannitol bromothymol blue agar (PEMBA) with the polymyxin and egg yolk concentrations remaining same as in MEYP and bromothymol blue replacing the phenol red as the indicator. The basal medium was also reformulated using (i) 0.1% w/v peptone, (ii) 1% w/v sodium pyrvate, so as to improve the egg yolk reaction, facilitate spore formation and reduce the spreading growth of *B. cereus* colonies and (iii)

phosphates to increase the buffering capacity of the medium. This was proposed to offer enhanced differentiation of *B. cereus* from mannitol utilizing and non-mannitol utilizing organisms. Also, addition of actidione was suggested for samples suspected with high levels of molds (Holbrook and Anderson, 1980).

Egg yolk reaction is the key diagnostic feature of *B. cereus* and can result in weak or lecithinase negative strains. Holbrook and Anderson (1980) propounded this is more likely to occur with KG, MEYP and PEMBA media. Strains of *B. cereus* with weak egg yolk reaction were known to be involved in food poisoning incidences. Hence, Gilbert and Taylor (1976) favoured the blood agar for primary isolation. Such strains can be identified by their typical colony morphology on PEMBA medium. However, weak or lecithinase negative strains need additional biochemical testing to confirm their identities.

Szabo et al. (1984) developed a new medium, polymyxin pyrvate egg yolk mannitol bromocresol purple (PEMPA) to overcome the problem of occurrence of atypical *B. cereus* colonies and inconsistent egg yolk reactions encountered on PEMBA. The replacement of bromothymol blue by bromocresol purple and a reduction in the incubation period to 18-24 h was claimed to give a more dependable diagnostic colony appearance and a consistent egg yolk reaction.

Rabinovitch and Meira de Vasconcellos (1987) designed a new medium namely methylene blue Mg^{2+}/Zn^{2+} glucose polymyxin agar (RVC) as a less expensive and alternative to MEYP and KG media. The medium contained polymyxin B polymyxin E (colistin) as selective agent. The nutrient rich composition of RVC promotes sporogenesis within 30-40 h. Isolates of *B. cereus* can then be differentiated by microscopy from *B. thuringiensis* on the basis of presence or absence of parasporal toxin-crystals. Zn^{2+} ions were added to enhance egg yolk reaction.

In later years, the use of non-selective blood free egg yolk agar containing inositol, mannitol and bromocresol purple was proposed (Hood et al. 1990). Weenk et al.

(1991) came up with the similar technique of overlaying MEYP medium with Tryptose sulphite cycloserine agar. The optimum incubation temperature and time was in the range of 30-37°C and 16-48 h. The egg yolk in the selective media confers some protection on sublethally injured cells, the use of resuscitation / rejuvenation of cells prior to selective media may be needed to achieve optimum recovery of viable, but injured cells of *B. cereus*.

Lancette and Harmon (1980) showed that the direct plating technique gave better repeatability and reproducibility than the MPN technique at both high and low levels of *B. cereus* contamination. For direct plating, blood agar is suitable for the isolation of large numbers of *B. cereus,* if foods were implicated in outbreaks (Gilbert and Taylor, 1976). Selective media like MEYP, PEMBA, KG and PEMPA are preferred for the enumeration of *B. cereus* in the presence of dominating background microflora in the samples. MEYP is predominantly used in North America, other ISO affiliated countries and also recommended by AOAC (Lancette and Harmon, 1980).

Szabo et al. (1984) compared the efficacy of selective media for the quantitative recovery of *B. cereus* and reported in the order of PEMPA > PEMBA > MEYP. The efficacy of PEMPA was attributed to a better suppression of background flora, the small sized colony and egg yolk reaction zone sizes observed. On the contrary, Holbrook and Andersen (1980) observed no significant difference in the recovery of *B. cereus* using McClung's egg yolk agar. The variations in the results can be attributed to the media composition, microflora of the foods tested or differences in the observation and interpretation of results.

The different selective and diagnostic systems used in plating media for the isolation of *B. cereus* are presented in a tabulated manner for an easy understanding.

Diagnostic characteristics of *B. cereus* on selective plating medium used in the isolation of this species

Plating medium	Diagnostic characteristic	Reference
Egg yolk agar	zone of halo around the colony due to lecithinase	McClung et al. (1946)
Bovine/horse blood agar (BA)	Haemolysis and Colony morphology	Gilbert and Taylor (1976)
Egg yolk-mannitol bromocresol Purple agar (EMBA)	zone of halo around the colony due to lecithinase; Purple coloured colony due to mannitol and BCP	Hood et al (1990)
Kendall's Bacillus cereus Medium (BCM)	zone of halo around the colony due to lecithinase; Purple coloured colony due to mannitol and BCP	Gilbert and Taylor (1976)
Citrate egg yolk polymyxin B lithium chloride agar (CELP)	zone of halo around the colony due to lecithinase	Donovan (1958)
Mannitol-egg yolk polymyxin B agar (MYEP)	zone of halo around the colony due to lecithinase	Mossel et al, (1967)
Columbia base-blood polymyxin B Agar (CBAP)	Haemolysis & Colony morphology	Kramer et al. (1982)
Kim and Goepfert (KG) agar	zone of halo around the colony due to lecithinase	Kim and Goepfert (1971b)
Polymyxin B-egg yolk mannitol bromothymol blue agar (PEMBA)	zone of halo around the colony due to lecithinase Peacock blue coloured colony due to mannitol and BTB	Holbrook and Andersen (1980)
Polymyxin B egg yolk mannitol bromocresol purple agar (PEMPA)	zone of halo around the colony due to lecithinase Purple coloured colony due to mannitol and BCP	Szabo et al. (1984)
Mannitol egg yolk polymyxin B agar (RVC)	zone of halo around the colony due to lecithinase	Rabinovitch and Meira de Vasconcellos (1987)

Appearance of *B. cereus* colonies on different media and culture conditions

Plating medium	colony appearance	Incubation Temp. (°C) and time (h)	Reference
BA	Raised grey-green, 3-8 mm ground glass surface, edge crenate to fimbriate, surface with zone of α- or β-hemolysis	30 and 20-24 35-37 and 24-48	Gilbert and Taylor (1976)
CELP	Crenate to fimbriate, surrounded by zone of halo around the colony due to lecithinase	30 and 18	Donovan (1958)
MEYP	Crenate to fimbriate, 3-6 mm, surface, zone of halo around the dark red coloured colony due to lecithinase	32 and 18-40 30 and 24-30	Mossel et al. (1967)
KG	Flat to slightly raised, crenate or irregular, 2-5 mm, surface, zone of halo around the dark red coloured colony due to lecithinase	35-37 and 16-24	Kim and Goepfert (1971b)
BCM	Crenate to fimbriate, raised colonies, 3-6 mm, surface, zone of halo around the purple coloured colony due to lecithinase	35-37 and 18	Gilbert and Taylor (1976)
PEMBA	Crenate to fimbriate, 3-5 mm, surface, raised, zone of halo around the peacock blue coloured colony due to lecithinase	37 and 24-48	Holbrook and Andersen (1980)
PEMPA	Crenate to fimbriate, 2-5 mm, surface,raised, zone of halo around the purple coloured colony due to lecithinase	37 and 18-24	Szabo et al. (1984)
RVC	Crenate to fimbriate, surface, raised, zone of halo around the blue coloured colony due to lecithinase	33-34 and 24-40	Rabinovitch and Meira de Vasconcellos (1987)

2.6.2 DNA-based methods

Foodborne diseases caused by pathogens are transmitted to humans either from their animal reservoir or through the process line. Detection and isolation of these organisms from food matrices are often difficult due to the high number of contaminating and indigenous microflora and a low number of the specific pathogenic organism. The conventional methods of isolation, which include selective enrichment and pre-enrichment, employed for a moderate sensitivity and specificity, are laborious and time-consuming. In the legislative and production control, microbiological examination of foods for the presence of pathogens is essential. This calls for the scope that exists for the rapid methods of isolation and detection of the organisms.

In a food microbiology laboratory, the rapid tests that are employed often are designed so as to be completed within limited time. According to Cox et al. (1987), the era of miniaturized techniques originated when Bachman and Weaver added concentrated inocula to small tubes of different bacterial media in the late 1940's. This led to the designing of commercial detection kits that are available in the market today. The basic principle of DNA-based tests is the ability of two single stranded DNA molecules under appropriate conditions to form a double stranded DNA by specific base pairing i.e. to hybridize. Based on this principle, three methods are routinely practiced:

i. Colony hybridization (Grundstein and Hogness, 1975)
ii. Single phase liquid hybridization assays (Curiale et al. 1990)
iii. Polymerase Chain Reaction (Saiki et al. 1988)

2.6.2.1 Colony hybridization

In the colony hybridization method, a food sample or an enrichment culture is spread on a nylon membrane or paper filter and incubated until visible colonies are present. These are processed to destroy the cells, remove cell substances and render fixed-single

stranded DNA for hybridization, by treatment with detergent and alkali or microwave treatment (Datta, et al. 1987). An enzyme or hapten labelled DNA probe, which constitutes part of the target DNA sequence is applied to hybridize to the sample DNA. Each signal on the filter corresponds to a positive identification upon direct spreading of samples. An essential step in the technique is the separation between labelled probe molecules bound to the target DNA and those that bind non-specifically to the filter. This is performed by stringency washing by fixing the target DNA to a solid support like filter.

2.6.2.2 Single phase hybridization

The decrease in the speed of hybridization because of stearic hindrance is overcome in this method. Here the hybridization between the target DNA and probe takes place in solution. The hybrids formed are captured afterwards and signals are developed. These assays are all targeted towards the variable regions of the ribosomal RNA (rRNA) and the high concentration of rRNA in the ribosomes of the growing bacteria, which then becomes part of the target and greatly enhances the speed of reaction (Meinkoth and Wahl, 1984).

2.6.2.3 Polymerase chain reaction (PCR)

In this method, a specific amplification of a defined target DNA is obtained by successive cycles of three steps namely (i) denaturation of template (sample DNA) to obtain single strand target, (ii) annealing of primers to the target sequences of template DNA and iii) polymerization of DNA starting from the primers with the help of thermostable enzyme *Taq* polymerase (Saiki et al. 1988). The number of amplicons is doubled in each cycle and all, but the fragments produced with the original target DNA as template will be of a fixed size, corresponding to the distance between the two primers.

Detection of the positive reaction can be done by visualizing the amplicon of expected size on running through agarose gel electrophoresis. Some primers anneal to different target sites and lead to the formation of products of similar size. Hence, the identity of the amplified fragment should be confirmed by hybridization to a specific DNA probe, digestion with a restriction enzyme or DNA sequencing of the amplicons. Another method of increasing the specificity of PCR is to combine two PCR methods (nested PCR), wherein in the first round, one set of primers is used to amplify DNA fragments from target DNA in the sample. The next set of primers is complimentary to an internal sequence in the correct PCR product resulted from the first round. Thus the formation of the desired amplicon is confirmed and false amplicons are ignored.

Though the PCR reaction is capable of detecting the DNA from a single organism, the volume of 5-10 µl of sample or culture taken for assay restricts the detection limit at approximately 3 $\log_{10}$ CFU/ml. Often, the pathogens are present in still lower numbers in the samples and direct detection by PCR is difficult to achieve at all times consistently. At the same time, the advantages of PCR-based methods are as follows:

1. PCR is a rapid technique with both high sensitivity and specificity. The assays can be made very specific by designing the appropriate primers targeting unique genetic markers.
2. Due to the relative high sensitivity, pre-enrichment and enrichment steps could be avoided or shortened significantly. The technique can be applied directly to the food samples, rather than plating followed by performing biochemical and serological tests.
3. It is useful for the detection and identification of organisms that cannot be cultured or difficult to culture by the routine laboratory procedures.

In contrast to the advantages, there are also limitations of PCR, which are listed below:

1 A beginner may find it difficult to successfully run the reactions and a certain extent of standardization/optimization with respect to various parameters is necessary at each laboratory level.

2 Presence of PCR inhibitors like bilirubin, bile salts, haemoglobin degradation products, polyphenloic compounds, proteinases, complex polysaccharides and fats restrict the activity of *Taq* polymerase activity and render the reaction non-functional.

3 Sample preparation and/or DNA extraction prior to the assay may become necessary, if the inhibitors are suspected to increase the specificity.

4 At times, due to the low number of target organisms in the sample, concentration technique or enrichment may be needed to increase the relative number of target organisms.

5 The method detects both live as well as dead organisms, hence not suitable for pasteurized, cooked and irradiated samples. However, useful in cases where the toxin secreted is heat stable.

6 Carryover of DNA from one reaction to another that contaminates the subsequent reactions may lead to false positive results.

Several oligonucleotide primers and probes for the detection of *B. cereus* and their toxins are being made use of in the recent days. Schraft and Griffiths (1995) developed three specific oligonucleotide primers for the detection of lecithinase positive *Bacillus* spp. The combined PCR-hybridization assay could detect *B. cereus* up to the limit of 1 CFU/ml of milk. Radhika et al. (2002) detected *B. cereus* by colony hybridization using the PCR-generated probe phospholipase. A broad range PCR methods was developed based on 16S rRNA sequence and successfully used for the

detection of *B. cereus* by spiking foods like milk, butter, cream, ham, etc., after a brief enrichment culture step (Nakano et al. 2004b)

Due to the close relatedness of the members of the *B. cereus* group, 16S rRNA sequence was found inadequate for the differentiation of *B. cereus* from the other related species. Jensen et al. (2005) explored the possibility of differentiating the *B. cereus* members using *gyrB* amplification, sequencing and restriction digestion. However, detection and differentiation of especially *B. cereus* from the *B. cereus* group was enabled by specific primers targeting sphingomyelinase, gyrase and *groEL* genes in combination with Restriction Enzyme digestion (Hsieh et al. 1999; Yamada et al. 1999; Manzano et al. 2003a, b; Chang et al. 2003). Later, a semi-quantitative method of detection of *B. cereus* group cells based on real time amplification of 16S rDNA sequence was performed on cardboard and paper samples spiked with spores and vegetative cells *of B. cereus* resulting in a detection level of 2 $\log_{10}$ CFU/g (Priha et al. 2004).

Similarly, primers for the detection of enterotoxin genes like *hblA*, *bceT* and *entFM* were proposed by different workers (Agata et al. 1995a; Asano et al. 1997; Mantynen and Lindstrom, 1998). A universal protocol for PCR detection of 13 species of foodborne pathogens was developed by Wang et al. (1997) which included primers for haemolysin gene of *B. cereus*. A method using fluorogenic probe-based PCR was developed for detection from nonfat dry milk targeting cereolysin and haemolysin genes with a detection limit of 2 $\log_{10}$ cells/g (Kim et al. 2000). A multiplex PCR which included 12 pairs of primers was designed for detection of five different enterotoxins and one emetic toxin and successfully analyzed by Yang et al. (2005). Enterotoxic *B. cereus* strains producing haemolytic and non-haemolytic toxins were detected by PCR from food and environmental samples, wherein the reference strain of *B. thuringiensis* also shared both the genes, thus indicating the potential risk associated with the use of *B.*

thuringiensis as a biopestcide agent (Oltuszak-Walczak et al. 2006). Strains of *B. cereus* producing cytotoxin were differentiated based on cytK-1 and cytK-2 (Guinebretiere et al. 2006). The haemolytic enterotoxin HBL was found to be broadly distributed among the species of the *B. cereus* group on performing molecular assay by primers (PrüB et al. 1999).

Cereulide was established as a putative product of non-ribosomal peptide synthetase (NRPS) with the help of primers targeting the NRPS sequence and comparing the amplicon sequence in other organisms like *B. brevis* and *B. subtilis.* Ehling-Schulz et al. (2005) were successful in developing PCR based on NRPS sequence targeting the cereulide synthetase gene (*ces*) for the characterization of emetic strains. Specific amplification and detection of *B. cereus* was performed by targeting neutral protease gene (*npr*) and further confirmed by blot hybridization (Bach et al. 1999). Primers based on a sequence characterized amplified region (SCAR), derived from a RAPD fragment were developed and used for the detection of emetic strains and differentiating from non-emetic strains (Nakano et al. 2004a).

The commonly used target genes and their Oligonucleotide primers used in the detection of *B. cereus* and their toxins are detailed in a tabular form.

Nucleotide sequence of specific primers and their target genes used in the detection of *B. cereus*

Target gene	Primer designation	DNA sequences (5'-3')	PCR product (bp)	References
hblA	HblA1/HblA2	5' GCTAATGTAGTTTCACCTGTAGCAAC 3' 5'AATCATGCCACTGCGTGGACATATAA3'	874	Mantynen and Lindstrom (1998)
Sph	Ph1/Ph2	5'CGTGCCGATTTAATTGGGGC3' 5'CAATGTTTTAAACATGGATGGATGCG3'	558	Hsieh et al. (1999)
bceT	ETF/ETR	5'TTACATTACCAGGACGTGCTT 3' 5'TGTTTGTGATTGTAATTCAGG3'	428	Agata et al. (1995a)
Ent FM	ENTF/ENTB	5'ATGAAAAAAGTAATTTGCAGG3' 5'TTAGTATGCTTTTGTGTAACC3'	1269	Asano et al (1997)
Hbl	BC1/BC2	5'CTGTAGCGAATCGTACGTATC3' 5'TACT'GCTCCAGCCACATTAC3'	185	Wang et al. (1997)
Pl-1	PLC-1/ PLC-2	5'GAGTTAGAGAACGGTATTTATGCTGC3' 5'CTACT'GCCGCTCCATGAATCC3'	413	Schraft and Griffiths (1995)
Ha-1	HA-1/HA-2	5'TGCGAGGTGAAATTCAACAA3' 5'GAACGCCCGAATATTGAGAA3'	489	Radhika et al. (2002)
Inverse repeat Inverse repeat	IS 231-P 231-1 IS 231-P 240	5'CATGCCCATCAACTTAAGAA3' 5'AAGGTTCTGGTGCAAAWAA3'	1650 850	Leonard et al. (1997)

2.6.3 Immuno-based detection

Immuno-based methods of detection of *B. cereus* and its toxins work on the principle of specificity of antigen-antibody reaction. The cell-surface antigens, spore antigens and flagellar antigens are made use of in the detection of the organism. Toxins are detected by the polyclonal antibodies directed against their antigenic nature. Antibody has been used for many years for serotyping since 1920s and 1930s. However, it was only after the introduction of radioimmunoassay and enzyme linked immunofluoroscent assay [ELISA] that the potential of antibodies in detection assays was understood (Engvall and Perlmann, 1971). Some of the widely used methods of immuno-detection are briefly presented below.

2.6.3.1 Latex agglutination and reverse passive latex agglutination

In latex agglutination (LA) method antibody-coated coloured latex beads or colloidal gold particles are used to test bacterial suspension. Visible clumping or precipitation is obtained, if the specific antigen is present. Though the technique is simple, it is not very sensitive and requires a minimum of 7 $\log_{10}$ CFU for a reaction. The toxins are detected by reverse passive latex agglutination (RPLA) in a microplate. Formation of a diffuse lattice by the soluble antigen is considered as a positive test. This may sometimes need multistep enrichment and is useful for quick serological confirmation of isolates from foods (Candish, 1991). The BCET of Oxoid uses RPLA method for detection. The presence of enterotoxin is detected by agglutination of latex particles sensitized with the antitoxin and detects the L_2 component of the enterotoxin (Griffiths and Schraft, 2002).

2.6.3.2 Enzyme linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay is the most widely used form of immunoassays performed. Commercially available ELISAs are designed as ‘sandwich assay’, whereby

antibody-coated solid matrices are used to capture the antigen from enrichment cultures and a secondary antibody conjugated with an enzyme (alkaline phosphatase or horseradish peroxidase) is added to form an antibody-antigen 'conjugate sandwich'. A colorimetric substrate is then added, which is cleaved by the enzyme to produce a colored product which is recorded visually or Spectrophotometrically. The detection of ELISA is usually about 4 $\log_{10}$ CFU/ml for bacterial cells and a few nanograms per ml or less for toxins. A fluorogenic substrate is sometimes added to enhance the sensitivity (Warburton et al. 1995). Flagellar antigen of *B. cereus* H1 was purified, characterized by immunoblot analysis and tested for sero-diagnostic assay by ELISA, thereby concluding a common antigen for *B. cereus* and *B. anthracis* (Murakami et al. 1993). TECRA-BDE visual immunoassay is a commercial kit having an ELISA format. The antibody used in the kit is active against two proteins, which are produced simultaneously with the enterotoxin (Griffiths and Schraft, 2002).

2.6.3.3 Immunomagnetic separation [IMS]

In this method, specific antibodies coupled to magnetic particles or beads are used to capture pathogens of interest from pre-enrichment media. IMS is analogous to selective cultural enrichment, whereby the growth of other bacteria is suppressed and the pathogen of interest is allowed to grow. It is also considered as a method to concentrate the sample after which the target organism is tested by plating onto selective media for further identification (Safarik et al. 1995).

2.6.3.4 Immunoprecipitation

This is basically a sandwich antibody test wherein the antibodies are labelled coloured latex beads or colloidal gold. These are simple assays requiring no washing and can be completed in 10 min after culture enrichment. (Warburton et al.1995).

2.7 INCIDENCE OF *B. CEREUS* IN A DIVERSE RANGE OF FOODS

The incidence of *B. cereus* is wide spread due to its ability to infect a broad range of foods, its endospore forming nature which is resistant to heat, dessication, disinfectants, ionizing radiations and UV light along with potential of growing in varied nutritional and environmental conditions. In recent times, the change in eating habits has increased the demand for minimally processed foods and new food formulations, which have broadened the scope for incidence of this organism. The organism can cause either of the foodborne illness namely diarrhoea and vomiting or may lead to spoilage of the foods involved. Studies have shown that milk and milk products are more commonly prone to contamination by *B. cereus*. The organism impairs the keeping quality of these products and its spores are able to attach to surfaces of pipelines, germinate and multiply in processing equipment. The organism can cause special types of spoilage like broken cream or sweet curdling of milk (Overcast and Atmaram, 1994).

2.7.1 Milk and milk products

Many psychrotrophic and mesophilic strains producing diarrhoeal enterotoxin were recovered from milk products (milk with rice, milk substitute, milk powder, pudding milk, flan and mousse) in a Chilean School Feeding Program (Larsen and Jorgensen, 1997; Reyes et al. 2007). Hammer et al. (2001) reported the incidence of *B. cereus* from a large dairy factory producing milk powder and examined for the production of toxin using a commercial kit and possibility of spores being passed from raw milk into finished products. Pirttijarvi et al. (1998) studied the incidence and origin of contamination by *B. cereus* in whey production line of a cheese factory and found biofilms being formed by colonizing the warm and hot areas of the pipeline equipments. Similarly, te Giffel (1996b) reported the incidence of *B. cereus* in dairy processing plants and concluded that the organism can be introduced through raw milk. Other sources like equipments can act as

potential reservoirs contributing to the post-processing contaminations. Becker et al. (1994) has reviewed the incidence of *B. cereus* in several infant and dried milk products, which includes the prevalence of the organism in skim and whole milk powder (Rodriquez and Barrett, 1986) with counts ranging upto 2 $\log_{10}$ CFU/g. Sallam et al. (1991) reported the presence of *B. cereus* in different milk and milk products like raw milk, pasteurized milk, ice cream, Domiati cheese and Kareish cheese samples with contamination level of 2 $\log_{10}$ CFU/ml.

2.7.2 Meat and meat products

Bacillus cereus is known to contaminate many meat and poultry products. Strains were detected from samples of chicken products obtained from retail stores in U.S. by conventional and PCR methods and were found to harbour different toxigenic traits for enterotoxin production (Smith et al. 2004). Troutt and Osburn (1997) reviewed and highlighted the potential microbiological hazards resulting from *B. cereus* associated with meat from cull dairy cows. Microbiological analysis of luncheon meat, chicken luncheon meat and chicken mince samples collected from Supermarkets in Cairo and Giza, Egypt revealed the presence of toxigenic strains of *B. cereus* up to the level of 2 $\log_{10}$ CFU/ml and lethal to mice (Abostate, 2006). Guven et al. (2006) screened samples of meat and meat products which included beef, meat mince, soudjouck and pastrami and detected low levels of *B. cereus* strains producing diarrhoeal toxin. However, the low count was attributed to the large number of background microflora and inhibitory substances produced by these which might have prevented the growth of *B. cereus*. Similarly, analysis of microbiological quality of raw meat balls marketed in Ankara, Turkey, showed the prevalence of *B. cereus* up to 2 $\log_{10}$ CFU/g and found that the hygienic quality of the samples was inadequate and could be a potential risk for public health (Kuplulu et al. 2003).

2.7.3 Legume and cereal-based foods

A variety of legume-based and cereal-based foods rich in starch are known to carry toxigenic strains of *B. cereus*. Emetic strains in particular are found to be more associated with farinaceous foods. Jang et al. (2006) reported occurrence of *B. cereus* in unhusked Korean rice and rice-products and tested for their enterotoxigenicity. Similarly cooked foods, rice noodles, wet wheat noodles, dry wheat noodles, spices, grains, legumes and legume products were found to carry diarrhoeal strains of *B. cereus* and were resistant to different antibiotics (Rusul and Yacoob, 1995). Some of the strains found to produce diarrhoeal toxin were isolated from fermented vegetable proteins used in seasonings in Nigeria, with counts ranging from 6.3 to 8.3 $\log_{10}$ CFU/g. However, their enzyme profile showed poor hydrolytic activity and hence the contribution to the fermentation of vegetable protein was found to be marginal (Oguntoyinbo and Oni, 2004). Diarrhoeal strains were detected in raw soy bean sprouts used as ingredients in cook-chilled products and were found to be eliminated after blanching (Kim et al. 2002, 2004).

2.7.4 Fruits and vegetables

Fruits and raw vegetables used in minimally processed foods act as reservoirs of *B. cereus* strains. Choma et al. (2000) recovered enterotoxigenic strains of *B. cereus* from cooked-chilled and pasteurized vegetable products like carrot, broccoli, split pea, courgette, potato purees and cooked leek. The initial count of the organism was <1 $\log_{10}$ CFU/g and ranged till 6-8 $\log_{10}$ CFU/g at 20°C before the appearance of spoilage in foods. Altayar and Sutherland (2006) analyzed samples of raw and processed vegetables like potatoes, carrots and lettuce at temperatures of 30 and 7°C and found to harbour strains of *B. cereus* with a mean spore count of 2.6 and 2.2 $\log_{10}$ CFU/g, respectively. It was concluded that among the total isolates obtained, the incidence of

emetic strains was found to be rare. Similarly, Ueda and Kuwabara (1998) detected several strains of *B. cereus* ranging up to an average count of 2 $\log_{10}$ CFU/g from fresh vegetables associated with outbreaks in Japan.

2.7.5 Other foods

Analysis of different food varieties like flours (rye and white), cheese and honey in Argentina showed prevalence of *B. cereus* indicating the significance of monitoring the samples for microbiological presence (Lurlina et al. 2006). Studies from the food samples of military canteen kitchens and catering systems of Germany Federal Armed Forces showed a high incidence of *B. cereus* (Ernst et al. 2001; Kleer et al. 2001). Strains of *B. cereus* were recovered from gnocchi, an Italian refrigerated processed food (REPFED) of extended durability and the growth was found to be inhibited by a combination of lactic acid and citric acid (Del Torre et al. 2001). Likewise sweet dumpling foods (flour-packed and rice-packed) and vegetarian foods whose components included soy bean, cereals, flour, vegetables, konjac, etc., manufactured and sold in Taiwan and traditional foods of Thai like *kapi* (shrimp waste), *nam-pla* (fish sauce), *pla-ra* (fermented fish), *pla-kem* (semi-dried salted fish), *phak-dong* (fermented vegetables), *yiaw-bong* (fermented fish cooked with spices and condiments), spices and rice flour were known to harbour *B. cereus* strains (Gasaluck et al. 1996; Du et al. 1996; Fang et al. 1999).

Various foods (milk, yeast, flour, pasta products, Chinese meals, cocoa, chocolate, bakery products, meat products, herbs and spices) in Netherlands and different fast foods (vegetable, rice, pasta, fish, meat and dairy dishes) on sales in Naples, Italy were known to be contaminated with *B. cereus* at 2 $\log_{10}$ CFU/g and incidence being more in fish foods (te Giffel et al. 1996a; Amodio-Cocchieri et al. 1998). Vegetative cells and spores of psychrotrophic strains of *B. cereus* were isolated from farms in The Netherlands which included samples of air, soil, grass, bedding, feed,

drinking water and milk. The psychrotrophs being able to germinate and grow at low temperatures can subsequently spoil the product faster than mesophilic types and thus have a significant impact on the keeping quality of milk (te Giffel et al. 1995).

2.7.6 Foods marketed in India

India being a sub-tropical country and known for a diverse range of food habits provides ample scope for the prevalence of *B. cereus*. Generally, a higher prevalence of the organism is observed in whole rice, cereals, pulses and food additives. Several researchers have reported the incidences from different foods over the years. Shankar et al. (1994) and Warke et al. (2000) detected psychrotrophic strains of *B. cereus* from ice cream samples of local brands in Mumbai. Similarly, samples of milk and milk products (*burfi, rasgulla, khoa, rasmalai*) collected from local vendors in Srinagar showed the prevalence of *B. cereus* at a level of 2 $\log_{10}$ CFU/g (Hussain et al. 2005). Some enterotoxigenic strains up to a level of 2 $\log_{10}$ CFU/g were found to be prevalent in milk and milk products like raw milk, *burfi*, skim milk powder, meat and meat products (chicken, mutton, butter chicken, chicken soup and mutton soup) in Ludhiana (Bedi et al. 2005) and non-enterotoxigenic strains were reported in *lassi*, butter and ice cream (Pillai et al. 1990; Pillai et al. 1993). Strains of *B. cereus* were obtained from variety of Indian foods like cooked and uncooked rice, spices, vegetables, pulses, oils and pasteurized and heat treated samples of milk (Seenappa and Kempton, 1981; Kamat et al. 1989; Rama Raju et al. 1989). The incidence was found to be highest in rice (particularly uncooked samples), pasteurized milk and dhal, while no strains were detected in freshly cooked rice samples (Shah et al. 1996). Roy et al. (2007) reported the incidence of several strains of *B. cereus* from six legume-based Indian fermented foods (*amrita, dhokla, dosa, idli, papad* and *wadi*) which could bring about spoilage of food. Varadaraj et al. (1992) reported the occurrence of *B. cereus* in various Indian snack and lunch

foods (*idli, vada, uppuma*, plain cooked rice, curd rice and *bisi bele bhath* with counts ranging from 2.6 to 4.2 $\log_{10}$ CFU/g.

2.8 BEHAVIOURAL PATTERN (MODELLING STUDIES) OF *B. CEREUS*

2.8.1 Culture media

Several studies have been undertaken in regard to the behavioural pattern of *B. cereus* in culture media. Spores surviving severe heat treatment undergo sub-lethal effects which result in greater nutritive requirements, modifications in optimum incubation temperatures and /or increased sensitivity to some inhibitors. Recovery conditions can significantly affect survival counts and the thermal resistance values (*D* and *z*) used to characterize the heat resistance of the strains. Leguerinel et al. (2000) studied the relationship between the apparent heat resistance of *B. cereus* spores in combination with the pH and NaCl concentration of the recovery medium using a simple 3-parameter model. The spores were heated at 95°C in phosphate-citrate buffer at pH 7 and NaCl concentration between 1 and 4 % (w/w) and *D*-values determined. A slight decrease in the *D*-value was observed with an increase in salt concentration. Results indicated the absence of interaction between NaCl concentration and pH of the recovery medium. Highest counts of heat-injured spores were obtained at near neutral pH and the same decreased as pH was lowered. The *z*-values were not affected by media pH. It was suggested that moderately low pH foods undergoing mild heat treatments enjoy an additional safety margin by virtue of their acidity levels, which needs to be further validated by experimental studies.

Gonzalez et al. (1997) studied the effects of addition of starch, glucose, NaCl, sodium citrate, monopotassium phosphate and disodium phosphate to the recovery medium on apparent heat resistance of *B. cereus* spores of strains ATCC 4342, 7004 and 9818. Sodium citrate, monopotassium and disodium phosphate at concentrations of

0.1% were effective inhibitory agents for heat injured spores. Increase in the salt levels from 0.5 to 4% resulted in a progressive decrease in spore recovery. The *z*-values ranged from 6.67 to 8.32 with a mean value of 7.56°C. Gonzalez et al. (1996) studied the influence of pH (range 7.6-5.4) of recovery medium on apparent heat resistance of 3 strains (ATCC 4342, 7004 and 9818) of *B. cereus.* Gonzalez et al. (1995) worked on the effects of recovery media and incubation temperature on the apparent heat resistance of 3 ATCC strains (4342, 7004 and 9818) of *B. cereus* spores in nutrient agar (NA), tryptic soy agar (TSA), plate count agar (PCA) and milk agar (MA) and temperature range of 15-40°C. Higher counts of heat injured spores were obtained on PCA and NA. No significant differences in heat resistance were observed with the different recovery conditions, except for strains 4342 and 9818, when MA was used as plating medium. The *z*-values obtained for the 3 strains studied under the different recovery conditions were similar. Couvert et al. (1999) proposed a 2-parameter model to assess the effect of heating medium and pH of recovery medium on the *D*-value and heat resistance of *B. cereus* spores. The model was suggested to be suitable for optimization of heat treatments of foods.

Similarly, Mazas et al. (1999a) determined the heat resistance characteristics (*D*- and *z*-values) of *B. cereus* spores of strains ATCC 7004, 4342 and 9818) at temperatures of 92-125°C in aqueous solutions of NaCl, LiCl, sucrose and glycerol over a_w range of 0.71-1.0. Heat resistance increased in the presence of NaCl, although effect varied between strains. LiCl had a less pronounced effect than NaCl at the same a_w values. Lowering of a_w values with glycerol (from 0.96 to 0.71) had little effect on *z*-values, but increased *D*-values by 30-60 folds depending on strain.

Mazas et al. (1995) studied the influence of sporulation media with respect to thermal resistance parameters of 3 strains of *B. cereus* (ATCC 4342, 7004 and 9818) in 4 sporulation media (nutrient agar supplemented with 1 ppm Mn^{+2}, fortified nutrient agar,

Angelotti medium and milk agar). In all the conditions, high rate of sporulation was obtained and a clear difference in the *D*-values of the spores produced in the 4 media was observed. The medium which yielded the most heat resistant spores and the magnitude with which the sporulation medium affected *D*-values were different for each strain. The *z*-values of the 3 strains were not influenced by the medium used to obtain spores. Using the same isolates, in another study, the researchers concluded that sporulation temperature in the range of 20-45°C did not have significant effect on *z*-values (Mazas et al. 1999a). Investigation into the effect of water activity on heat resistance of *B. cereus* spores using a 4-parameter model showed a linear relationship between log *D* and a_w (Gaillard et al. 1998). The effect of salt stress and physiological state on thermotolerance of *B. cereus* (ATCC 10987 and ATCC 14579) at 50°C were quantified using linear and non-linear microbial survival models which resulted in the distinct tailing pattern of strain ATCC 10987. The effect was attributed to the presence of sub-population of spores present in the sample analyzed (Besten et al. 2006).

Olmez and Aran (2005) developed models to describe the growth kinetics of *B. cereus* at different temperatures, pH and concentrations of sodium lactate and NaCl. The natural logarithms of kinetic parameters (lag phase duration and growth rate) were used in reduced polynomial models and predicted data was compared with that of Pathogen Modeling Program (ARS, USDA). All of the studied variables had a significant effect on growth kinetics. In another study, using a Weibul frequency distribution function, an antibacterial activity of thymol and cymene against *B. cereus* strains (INRA-AVTZ415 and INRA-AVZ421), in the exponential growth phase suspended in pH 7 HEPES buffer at 30°C, it was found that the compounds could be of potential use in preservation of minimally processed foods (Delgado et al. 2004). A response surface model was developed to describe the effects of temperature (20-40°C), pH (4.5-6.5) and a_w (0.94-0.99) on germination of *B. cereus* ATCC 11778 spores. The germination was affected (*P*

<0.05) by interactions of a_w with temperature and pH, and by temperature in its quadratic term (Sinigaglia et al. 2002).

The effect of 4 independent variables namely temperature (10-40°C), pH (4.0-6.0), a_w (0.89-0.99) and ethanol concentration (0.5-2.0% v/v) on the growth / no growth boundaries was investigated. Results showed that temperature, pH, and a_w significantly affected the location of the growth/no growth boundaries. *Bacillus cereus* was unaffected by the combined variables, as long as they were above the minimum values for growth (Lanciotti et al. 2001). Leguerinel and Mafart (2001) developed a model to describe the effects of pH (4.0-7.0) and organic acids (L-glutamic, malonic, malic, lactic, succinic, adipic and acetic acids and glucono-delta-lactone) on the heat resistance of *B. cereus* CNRZ 110 spores. At pH 7.0, when 99.6% of acetic acid was dissociated, the *D*-value of spores revealed that this dissociated acid form exerted a protective effect on spore heat resistance. Sutherland et al. (1996) constructed a 4-factor growth model using vegetative cells of *B. cereus* with controlling factors of temperature (10-30°C), pH (4.5-7.0), NaCl (0.5-1.5%) and CO_2 (10-80% v/v). The model predictions gave accurate doubling time estimates on a variety of foods including meat, milk and carbohydrate-based products.

2.8.2 Effect of cold temperatures

Foegeding and Berry (1997) screened food and clinical isolates for their ability to grow at cold temperatures using fluid or solid nutrient media or milk incubated at 10, 7 or 5°C. Few isolates could grow in brain heart infusion (BHI) at 7°C, if previously adapted to this temperature (7°C). Also, both the food and clinical isolates showed a cold adaptation response which needs to be considered when modeling growth in foods or in assessing shelf life. The growth of 3 dairy isolates of *B. cereus* was determined in relation to inoculum size and storage temperatures (4, 8 and 20°C) and was found that the survival of these for extended periods could pose health hazard (Little and Knochel, 1994).

Another study involving modelling at low temperature by response surface was performed to determine the effects and interactions of a_w (0.965-0.995), pH (5.8-8.0), temperature (6-38°C), glucose concentration (0-1.8%) and starch concentration (0-0.625%) on growth and toxin production of a psychrotrophic strain of *B. cereus*. Results indicated that the a_w and temperature had the greatest influence on both, growth and toxin production and the predicted values were in good agreement with experimental values (Baker and Griffiths, 1993).

2.8.3 Food systems

Studies on behavioural pattern of *B. cereus* have included both vegetative cells and spores in different food systems. Byrne et al. (2006) designed a thermal treatment(s) for pork luncheon roll, which could destroy *B. cereus* vegetative cells and spores. The *D*-values for *B. cereus* vegetative cells ranged from 1.0 min (60°C) to 33.2 min (50°C) and for spores from 2.0 min (95°C) to 32.1 min (85°C). The *z*-values were calculated to be 6.6 and 8.5 °C for spores and vegetative cells, respectively. The *D*-values suggest that a mild cook of 70°C for 12s and 36s at 105°C for vegetative cells and spores, respectively, would achieve a 6 log reduction. The effect of acidification of carrot extract with different acidulants (Malic, citric, ascorbic, lactic and hydrophobic acids) was studied by Moussa-Boudjemaa et al. (2006). Acidification used for carrot with any type of acidulant extract reduced the *D*-values obtained for spores. Significant decreases in survival counts and apparent *D*-values were observed when injured spores were recovered using the usual recovery medium. Likewise, the influence of pH on the heating medium (milk, tomato, rice and beans) on thermal resistance of spores of *B. cereus* showed that acidification reduced the *D*-values and found a linear relationship between log *D*-values and pH, which made it possible to develop an equation to predict approximately the changes in heat sensitivity (Mazas et al.1998).

Using Bigelow model, Leguerinel et al. (2005) described the effect of heating and recovery pH and a_w on heat resistance of *B. cereus* ADQP 407 in cream chocolate. Loss and Hotchkiss (2002) investigated the effect of CO_2 on thermal inactivation of microorganisms during pasteurization using Weibull function. The CO_2 levels ranging from 44 to 58 mM added to whole milk reduced the number of surviving *B. cereus.* Penna et al. (2002) studied the growth kinetics of germinated cells from activated spores of *B. cereus* inoculated into cooked white rice and milk at different temperatures for control samples and samples containing nisin. No development of *B. cereus* was observed in milk + nisin incubated at 40°C for 12 h. Studies on the same line for spores of *B. cereus* ATCC 1479 in cooked white rice, rice water (pH 6.7), phosphate buffer (pH 7.0) and milk supplemented with nisin showed a mean reduction of 40% in *D*-values over a range of 80 to 100°C (Penna and Moraes, 2002).

An investigation by Fernandez et al. (2001) showed that germination of spores of *B. cereus* (INRA AVZ421 and INRA TZ415) was higher after non-isothermal heat activation than after isothermal activation. Wandling et al. (1999) determined *D*-values for spores of *B. cereus* T in skim milk supplemented with nisin concentration (0, 2000 and 4000 IU/ml) and found significant decrease in the apparent *D*-value between the control and treated. The results suggested that spore control is likely due to enhanced sensitivity of spores to heat and the presence of residual nisin in the recovery medium that could prevent outgrowth of survivors. Heat resistance of *B. cereus* (ATCC 7004, 4342 and 9818) heated in different types of milk (skim, whole and concentrated skim milk) containing stabilizing additives (sodium citrate, monopotassium phosphate or disodium phosphate, 0.1%) and cream was investigated by Mazas et al. (1999a). Of the stabilizing additives tested, only sodium citrate and sodium phosphate increased the heat resistance of strain 9818. It was concluded that the differences in the heat resistance observed could be due to a pH effect, rather than to the difference in the

substrates in which spores were heated. The *z*-values were not significantly modified by the milk composition.

2.9 MOLECULAR BASIS FOR DIVERSITY OF PATHOGENIC/VIRULENCE TRAITS IN *B.* CEREUS

Bacillus cereus comprises closely related Gram positive bacteria that exhibit highly divergent pathogenic properties. The group consists of *B. cereus*, *B. thuringiensis, B. anthracis, B. wiehenstephanensis, B. mycoides* and *B. pseudomycoides*. There has been a long drawn confusion over the taxonomic status of the 3 species (*B. cereus, B. thuringiensis and B. anthracis*) since decades, which still remains disputed among the researchers (Radnedge et al. 2003). Whole genome DNA hybridization has been of not much help (Kaneko et al. 1978), while conventional markers of chromosomal diversity, such as 16S and 23S rRNA genes are identical (Ash et al. 1991b). Comprehensive studies using a diverse range of techniques including genomic mapping (Carlson et al.1996), pulsed-field gel electrophoresis of chromosomal DNA (Carlson et al. 1994), multilocus enzyme electrophoresis (Helgason et al. 2004), BOX-PCR fingerprinting (Kim et al. 2001), amplified fragment length polymorphism analysis (Ticknor et al. 2001) and multilocus sequence typing (Helgason et al. 2004) have shown extensive similarities and very few differences among the isolates belonging to the *B. cereus* cluster. Despite these arguments, separate species status has been maintained, because of their pathogenic features. The *B. cereus* group comprises members that have most likely evolved from a saprophyte or insect gut commensal common ancestor, principally by asexual process. Eight lineages have arisen, each of which appears to have global distribution, although the presence of unassigned *B. cereus* genotypes represented by ST-30 and perhaps ST-38 suggests that more exhaustive sampling would identify further lineages.

The paradigm shift from the use of biological phenotypes as markers to using molecular markers such as SNPs (single nucleotide polymorphisms), indels (insertions and deletions) and tandem repeats which leverage on the genome sequence data have made the evolution study of the bacteria more convincing than earlier. The degree of relatedness of members of the *B. cereus* group is evident on comparing the genome sequences of two non-pathogenic *B. cereus* strains (ATCC 14579 and ATCC 10987) with that of pathogenic *B. cereus*. The wide range of differences in phenotypes and pathological effects are attributed to the factors encoded on extra-chromosomal elements like large toxin producing plasmid pXO1 in *B. anthracis*. An analysis of the genomes of *B. cereus* and *B. anthracis* showed a small subset of genes unique to either species, most of which are annotated as hypothetical. The majority of these genes were found located at the terminus of replication, indicating that genome plasticity mostly occurs in that region, as observed earlier for other microbial groups. It is also reported that, in many cases, genes found at a specific position in one genome were replaced with others at the corresponding loci in another. These regions were often the result of insertion/deletion events of mobile genetic elements like phages, transposons, IS elements or metabolic adaptations (Suyama and Bork, 2001).

Additionally, events like a xylose utilization operon replacing a function of nitrate reduction and the mobility of pXO1 gene within the *B. cereus* group as evidenced by DNA microarray and comparative genome hybridization (CGH) underscores the role of insertions and deletions in the evolution of *B. cereus* group (Rasko et al. 2005). Comparative analyses suggest that major differences among members of the *B. cereus* group might represent fine alteration in gene expression, rather than the sequence level divergence or gene content. *PlcR* is a pleiotropic transcriptional regulator that upregulates the expression of more than 100 genes in *B. cereus* through binding to an upstream palindromic motif (Gohar et al. 2002). Even though genes for virulence factors

like collagenase, phospholipases, haemolysins, proteases and enterotoxins are present in *B. anthracis*, a non-sense point mutation in the *PlcR* gene is responsible for an abolition or dramatic reduction in their expression, thus indicating the significance of mutations (Gohar et al. 2002).

2.9.1 Molecular epidemiology

On the basis of established knowledge of microbial genetics one can distinguish three major natural strategies in the spontaneous generation of genetic variations in bacteria. These strategies are: (i) small local changes in the nucleotide sequence of the genome, (ii) intragenomic reshuffling of segments of genomic sequences and (iii) the acquisition of DNA sequences from another organism. The three general strategies differ in the quality of their contribution to microbial evolution. Besides a number of non-genetic factors, various specific gene products are involved in the generation of genetic variation and in the modulation of the frequency of genetic variation. The underlying genes are called evolution genes. They act for the benefit of the biological evolution of populations as opposed to the action of housekeeping genes and accessory genes which are for the benefit of individuals (Arber, 2000).

In general, strains of *B*. *cereus*, *B*. *thuringiensis* and *B*. *anthracis* are known to be highly similar phenotypically and genotypically. Hence, they are regarded as belonging to the same *B*. *cereus* cluster. Unlike eukaryotes, which evolve principally through the modification of existing genetic information, bacteria have obtained a significant proportion of their genetic diversity through the acquisition of sequences from distantly related organisms. Horizontal gene transfer produces extremely dynamic genomes in which substantial amounts of DNA are introduced into and deleted from the chromosome. These lateral transfers have effectively changed the ecological and pathogenic character of bacterial species. The addition of genes through duplications

and horizontal transfer may also contribute indirectly to the emergence of opportunistic pathogens that are versatile with respect to environment and hosts. All the members of *B*. *cereus* group are known to have a very efficient conjugation system which facilitates the exchange of genetic material between the strains (Kimura, 1980).

As bacterial genomes can maintain only a finite amount of information against mutation and loss, chromosomal deletions will serve to eliminate genes that fail to provide a meaningful function, i.e. the bulk of acquired DNA as well as superfluous ancestral sequences. Hence, bacterial genomes are sampling, rather than accumulating sequences, counter-balancing gene acquisition with gene loss. As a result gene transfer can refine the ecological niche of a microorganism, which will, in effect promote bacterial speciation (Ochman et al. 2000). Another source of genome variability that plays an important role in prokaryotic genome evolution is lateral gene transfer (LGT), which brings new genes into a genome and provides a means for rapid adaptations to changing demands on an organism. For example, acquisition of virulence determinants on pathogenicity islands appears to play a major role in pathogen evolution (Boucher et al. 2003).

Point mutations, genomic rearrangements and horizontal gene transfer are driving forces in microbial evolution (Arber, 1993). While point mutations lead to 'slow' evolutionary development, the acquisition and excision of large genomic fragments quickly generate new variants of strains by means of 'genetic quantum leaps'. Phages, plasmids and pathogenicity islands are involved in those processes of fast evolutionary movement. The acquisition of new genes following horizontal gene transfer either directly by transformation with naked DNA, transduction with phages, or the uptake of plasmids or chromosomal fragments by conjugation, results in the generation of new variants of pathogens. The generation and acquisition of pathogenicity islands may be

directly associated with higher mutation rates and recombination efficiencies in pathogens (Falkow, 1996).

For most bacterial pathogens virulence is a multi-factorial process requiring two general classes of determinants. The first encompasses genes that participate in physiological processes necessary for survival in host and non-host environments, and these genes are generally found in both pathogenic and non-pathogenic organisms. The second class of virulence genes specifies traits that are unique to pathogens, and not surprisingly, these genes are rarely detected in non-pathogenic organisms. Incorporation of a pathogenicity island can, in a single step, transform a normally benign organism into a pathogen.

2.9.2 Gain and loss of pathogenicity

The occurrence of pathogenicity islands raises several questions about the evolution of virulence in bacteria. First, what advantage does the incorporation of virulence genes into the chromosome provide over retaining them on episomes? Although many virulence determinants occur on extrachromosomal DNAs, integration into the chromosome circumvents the need for autonomously replicating elements. This also lowers the probability that a given gene will be eliminated, because episomes must be under continuous selective pressure to be maintained and the spontaneous elimination of chromosomal genes is not as high. However, plasmid integration can result in the activation of chromosomal loci or in the alteration of the expression of plasmid-encoded genes. Three factors determine the virulence role of pathogenicity islands namely (i) the genes within the island, (ii) the status of the recipient microorganism and (iii) features of the host that promote the progression of disease. The acquisition of pathogenicity islands offers a rapid method of evolving novel functions, however, the introgressed sequences, even when they encode their specific regulators as part of complete

functional units, must interact with rest of the genome. The deletion of a pathogenicity island may represent a regulatory mechanism to control expression of virulence genes (Groisman and Ochman, 1996).

Despite a consistent correlation between genome size and the obligate association with host cells, genome reduction is not simply an adaptive response to living within hosts. Instead, the trend towards large scale gene loss reflects a lack of effective selection for maintaining genes in these specialized microbes. Because the host presents a constant environment rich in metabolic intermediates, some genes are rendered useless by adoption of a strictly symbiotic or pathogenic life style. These superfluous sequences are eliminated through mutational bias favouring deletions, a process apparently universal in bacterial lineages. All of the fully sequenced small genomes display a pattern of loss of biosynthetic pathways.

The relationships between members of the *B. cereus* group are non-linear and complex, likely resulting from cycles of isolation and niche expansion facilitated, at least in part, by horizontal gene transfer mechanisms. While the germination of *B. anthracis* spores or its vegetative growth may be limited to nutritionally rich environments like that found in a mammalian host, the rapid death of the host resulting from vegetative growth would limit the opportunity for genetic exchange and would result in the homogeneity observed in sequenced strains of this species. In contrast, the capacity for vegetative growth outside an infected host or non-lethal infection provides an opportunity for genetic exchange and niche expansion. The sequences of the two *B. cereus* group members presented here provide fertile ground to study the evolution of host range and virulence (Hernandez et al. 1998).

2.10 *BACILLUS CEREUS* IN PRESENT SCENARIO OF FOOD SAFETY

The symptoms of *B. cereus* infection are usually mild and self-limiting and would not generally need treatment. Since it is invariably isolated from foods and can survive extended storage in foods, it is not practically feasible to eliminate the spores when they are in low numbers. Control against food poisoning should aim at preventing germination of spores and minimizing the growth of vegetative cells. To attain this, foods should be maintained above 60°C or rapidly and efficiently cooled to less than 7°C and be thoroughly reheated before serving (Griffiths and Schraft, 2002).

Bacillus cereus forms an important organism among foodborne pathogens. The endospore forming ability and property of biofilm formation have augmented the need to address the incidence of strains with toxigenic traits. The emergence of psychrotrophic strains with varying heat resistance and behaviour of the organism in a wide variety of evolving food systems assumes significance in the light of the increased prevalence of the organism and under-reporting of the illnesses, because of the mild and self limiting nature of the manifestations.

3.0 OBJECT AND SCOPE OF THE STUDY

Bacillus cereus is an opportunistic foodborne pathogen that contaminates a wide variety of foods. It is wide spread in nature and frequently isolated from soil and growing plants. It was for the first time that this species was isolated from air in cowshed by Frankland and Frankland in 1887. Morphologically, culturally, biochemically and genetically very close to *Bacillus anthracis* and *Bacillus thuringiensis*, B. cereus is also included in the same group and, in general, named as *B. cereus* cluster or group. The organism is present in the natural surroundings and gains an entry into food system through water and other raw materials used in the preparation of food. The study of the organism has gained significance due to its endospore forming nature, which are resistant to heat, nutrition depletion, desiccation and other such factors.

There are mainly two types of illness caused by *B*. *cereus* in human beings namely diarrhoeal and emetic types. The diarrhoeal type is caused by different enterotoxins and characterized by symptoms of profuse diarrhoea and abdominal cramps. The general symptoms for emetic type are nausea and vomiting. The emetic type of illness is found to be more associated with starch-based foods like rice. The diarrhoeal enterotoxins are heat labile and emetic toxin is heat stable. The clinical manifestations in both the cases are mild and self-limiting, last for 2-3 days and most often do not need medical intervention. Hence, most of the cases go unreported and not documented for incidence. Isolates of *B. cereus* has been isolated from foods by conventional protocols using a variety of selective media. The recent studies reporting the foodborne illness being caused by all the members of *B. cereus* have made the specific identification of *B. cereus* all the more important. In this context, a study of prevalence of the native isolates of *B. cereus* in different foods would give an insight into the presence of toxigenic isolates in food samples analyzed. The use of PCR-based

markers with primers specific for species and toxin genes has been gaining significance due to their specificity in identification and rapidity.

India being a vast and subtropical country with plethora of food varieties being prepared at household and commercial level provides an ideal situation for an organism like *B*. *cereus* to survive and grow leading to health hazards. The cultural diversity and heterogeneity factors in India being reflected in foods and food consuming habits of people combined with the movement of foods across different countries by way of exports and imports have further necessitated the need for the study of *B. cereus* given its potential to pose risk factors to public health.

In the background of Indian scenario, the present Ph.D. programme attempts to assess the prevalence of toxigenic and non-toxigenic isolates of *B. cereus* in selected foods being sold in local markets based on biochemical and molecular characterization. Along with this, a phylogenetic relationship of the selected native isolates with those documented earlier in regard to their nucleotide sequence of selected toxin genes was also the focal point of study. The ability of *B*. *cereus* to exist in dual phase and the majority of Indian traditional foods being heat processed, it was felt necessary to assess the thermal inactivation profile of *B*. *cereus*. Another focal point of present programme aimed at predicting the behaviour of a selected toxigenic isolate in terms of two important phases of growth cycle. At the same time, the behavioural study with the same toxigenic isolate was extended to selected food matrices. The overall objectives of present study were as follows:

- Diversity of isolates of *B. cereus* in selected foods with a focus towards PCR-based detection and analysis for toxin potential
- Extrinsic and intrinsic factors influencing the growth of *B. cereus* (vegetative cells and spores)
- Behavioural pattern of spores and vegetative cells of *B. cereus* in cereal-legume based and milk based foods towards development of predictive models

4.1 PREVALENCE OF POTENT TOXIGENIC / PATHOGENIC ISOLATES OF *BACILLUS CEREUS* IN INDIAN TRADITIONAL FOODS

It has been very well established that *Bacillus cereus* has the ability to contaminate a wide variety of foods and hence is widely distributed in the ecosystem. With the advent of globalization and open markets, combined with the more stringent food laws and regulations, the study of prevalence of *B. cereus* has assumed significance. Isolation by conventional methods and characterization would give an insight into prevalent status of the organism and their toxigenic potential. Conventional techniques have limited scope in microbial typing, since genotypic differences do not necessarily encode differences in phenotypic traits. Randomly Amplified Polymorphic DNA (RAPD, a DNA based approach for typing the organisms has been proved useful in assessing the degree of phenotypic relatedness among the strains and assist in differentiating the dissimilar isolates.

This experimental chapter aims at assessing the prevalence of *B. cereus* isolates in a range of foods by conventional selective plating protocols, followed by identification by morphological, cultural and biochemical characteristics. The study of species specific detection of the isolates and genotypic detection of toxigenic traits has been undertaken using specific primers. A phylogenetic relationship among the selected isolates based on the nucleotide sequences and molecular diversity of the identified isolates has been presented. Similarly, relatedness among native isolates based on isolation sources and potent toxigenic traits has also been attempted through the use of RAPD markers.

4.1.1 MATERIALS

All glasswares, media and other materials used in the present study were either wet sterilized or dry sterilized. Wet sterilization was carried out at 121°C for 20 min in an autoclave and dry sterilization at 180°C for 4 h in a Hot Air Oven. All bacteriological media used were those of dehydrated media procured from Hi-Media Lab., Mumbai,

India (Hi-Media, 2003). The media were prepared as per manufacturer's instructions. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

The specific requisites in respect of PCR specific reagents and fine biochemicals such as *Taq* polymerase, base pair ladder molecular markers, buffers, reagents, agarose, gel stain (ethidium bromide) used in this experimental study have been of molecular biology grade and obtained from Sigma-Aldrich, Bangalore, India, Bangalore Genei, Bangalore, India and Sisco Research Laboratories, Mumbai, India.

4.1.1.1 Food samples

A total of 65 food samples were collected from various sale points in the local markets of the City of Mysore, Karnataka State, India. The samples consisted of the following:

i. traditional fast foods based on processed rice and wheat-based foods added with vegetables, spices and seasonings with common names of *pani puri*, *bhel puri*, *churmuri*, *masala puri, sev puri* (**n = 30**)
ii. traditional concentrated and sweetened milk sweets like *peda*, *burfi*, *malai sandwich*, raw milk and ice cream (**n = 10**)
iii. fried wheat-based and vegetable stuffed foods like bread sandwich, cutlets and traditional food by name of *samosa* (**n = 10**)
iv. spiced cooked rice-based traditional foods of common names of *bisibele bhath*, *lemon rice*, *ghee rice* and *vegetable pulav* (**n = 15**)

All the samples were collected under sterile conditions and avoiding any external contamination in appropriate pre-sterilized containers (like screw-capped tubes, sterile polypropylene pouches and glass beakers and/or conical flasks). The collected samples were placed in an ice-box, brought to the laboratory for analysis within 30 min of collection and immediately subjected to analysis.

4.1.1.2 Reference culture

As a reference culture, strain of *B. cereus* F 4810 was obtained through the courtesy of Dr. J.M. Kramer, Central Public Health Laboratory, London, United Kingdom and food isolates of *B*. *cereus* CFR 1410 and 1462 (Radhika et al. 2002) maintained in the culture collection stock of this Department were included as positive controls in the experimental protocols. The cultures were maintained at 6°C on brain heart infusion (BHI) agar slant. Prior to use in experimental trials, isolates were propagated successively twice in BHI broth for 24 h at 30°C.

4.1.1.3 Diluent and selective agents

4.1.1.3.1 Normal saline

The diluent used was 0.85% normal saline, which was dispensed in requisite quantities in suitable glass containers and autoclaved.

4.1.1.3.2 Polymyxin B sulphate selective supplement

This is an antibiotic supplement and is an essential requirement for the use of *Bacillus cereus* agar base in the isolation protocols. The ready-to-use vial with 50000 Units was reconstituted in 2 ml sterile Milli Q water, gently agitated for complete mixing of content in water and then aliquots of the supplement were used to give the requisite final concentration.

4.1.1.3.3 Egg yolk suspension

A quantity of 25 ml of distilled water was taken in a clean Erlenmeyer conical flask containing glass beads, each of 2-3 mm diameter, plugged with cotton and autoclaved. To this sterilized water (tempered to ambient temperature) was added yolk content of one surface cleaned egg under aseptic conditions. The contents of flask were swirled well to make a uniform suspension of yolk. The prepared

suspension was used freshly in the experiment and/or stored at 4°C for a maximum period of 48 h.

4.1.1.4 Gram stain (Acuff, 1992)

Crystal violet

Crystal violet	2.0 g
Ethyl alcohol (95%)	20.0 ml
Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Crystal violet and ammonium oxalate were dissolved in ethyl alcohol and distilled water, respectively and the two solutions were then mixed. The prepared stain was filtered and stored in a clean and dry glass stoppered bottle.

Lugol's iodine (mordant)

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300.0 ml

Safranin (counter stain)

Safranin-0	2.5 g
Ethyl alcohol (95%)	100.0 ml

Ten ml of the above stock solution was mixed with 90 ml of distilled water for use as counter stain.

4.1.1.5 Spore stain (Acuff, 1992)

Malachite green (5%)

Malachite green	5.0 g
Distilled water	100.0 ml

Safranin

Safranin-0 (2.5% solution in 95% ethyl alcohol)	10.0 ml
Distilled water	90.0 ml

4.1.1.6 Delta endotoxin crystal stain

Amido Black

Napthol blue black in 1.5 g was dissolved in 50 parts of 98% methanol, 40 parts distilled water and 10 parts acetic acid. Preparation stored for 3 days prior to use.

Carbol Fuchsin

Basic Fuchsin of 1 g was dissolved in 10 ml of 95% ethanol and mixed with 5 g of phenol, dissolved in 90 ml of distilled water. Use at 30% in aqueous solution.

4.1.1.7 Bacteriological media

4.1.1.7.1 Bacillus cereus agar base

The medium composition is as follows (g/l):

Peptic digest of animal tissue	1.0
Mannitol	10.0
Sodium Chloride	2.0
Magnesium sulphate	0.10
Di-sodium Phosphate	2.50
Monopotassium phosphate	0.25
Sodium Pyrvate	10.0
Bromo Thymol Blue	0.12
Agar	15.0
Final pH 7.2 ± 0.2	

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate quantities into Erlenmeyer conical flasks and/or test tubes (18 x 150 mm) plugged with cotton and autoclaved.

To 95 ml quantity of pre-sterilized and molten *Bacillus cereus* agar base medium tempered to 45°C were added polymyxin B sulphate (4.1.1.3.2) at a concentration of 100 IU/ml and 5% of egg yolk suspension (4.1.1.3.3), mixed well and poured into sterile petri plates at the rate of 20 ml per plate. Poured plates were allowed to solidify at ambient temperature and stored at the same temperature for use in experiment within 24 h of pouring the plates.

4.1.1.7.2 Blood agar

To 100 ml of molten, autoclaved and tempered sterile 1% plain agar, 2.5% of freshly prepared defibrinated sheep blood was added, mixed well and poured in sterile petri plates. The poured plates were allowed to solidify, then kept at ambient temperature and used in the experiment within 24 h of preparation.

4.1.1.7.3 Brain heart infusion (BHI) broth and agar

The medium composition is as follows (g/l):

Peptic digest of animal tissue	10.0
Calf brain, infusion	12.5
Beef heart infusion	5.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
Final pH 7.2 ± 0.2	

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate quantities into Erlenmeyer conical flasks and/or test tubes (18 x 150 mm) plugged with cotton and autoclaved.

BHI agar medium was prepared by using agar at a strength of 1.5% in BHI broth medium. The agar medium was boiled to dissolve the agar, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity as well as in 10 ml amounts in test tubes (18 x 150 mm) plugged with cotton and autoclaved. For the purpose of making slants, after autoclaving, tubes containing BHI agar were kept in a slanting position, so as to have 1" butt and remaining slants.

4.1.1.7.4 Hugh Leifson medium

The medium composition is as follows (g/l):

Peptone	2.0
Sodium chloride	5.0
Glucose	10.0
Dipotassium phosphate	0.3
Bromo thymol blue	0.05
Agar	2.0
Final pH	7.2 ± 0.2

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 3 ml amounts in the test tubes (12 x 75 mm) and autoclaved.

4.1.1.7.5 MR-VP medium -Buffered glucose broth

The medium composition is as follows (g/l):

Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH	7.2 ± 0.2

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 10 ml amounts in the test tubes (18 x 150 mm) and autoclaved.

4.1.1.7.6 Nitrate broth

The medium composition is as follows (g/l):

Beef extract	3.0
Peptone	5.0
Potassium nitrate	1.0
Final pH	7.2 ± 0.2

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 5 ml amounts in the test tubes (18 x 150 mm) and autoclaved.

4.1.1.7.7 Nutrient broth and agar

The medium composition is as follows (g/l):

Peptic digest of animal tissue	5.0
Beef extract	3.0
Sodium chloride	5.0
Final pH	7.2 ± 0.2

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in Erlenmeyer conical flasks and /or test tubes (18 x 150 mm) and autoclaved.

Nutrient agar medium was prepared by using agar at a strength of 1.5% in nutrient broth medium. The agar medium was boiled to dissolve the agar, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity as well as in 10 ml amounts in test tubes (18 x 150 mm) plugged with cotton and autoclaved. After autoclaving, tubes containing nutrient agar were kept in a slanting position, so as to have 1" butt and remaining slants.

4.1.1.7.8 Nutrient agar – gelatin medium

This was prepared by boiling appropriate quantity of nutrient agar (4.1.1.7.7) containing gelatin at 12% level, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity, plugged with cotton and autoclaved. The sterilized medium was tempered to 45°C and poured in to sterile petri plates at the rate of 20 ml per plate, allowed to solidify and placed at ambient temperature for use in experimental trials within 24 h of pouring the plates.

4.1.1.7.9 Nutrient agar – milk medium

This was prepared by uniformly mixing 10 ml of 10% reconstituted skim milk with 90 ml quantity of melted nutrient agar (4.1.1.7.7), dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity, plugged with cotton and autoclaved. The sterilized medium was tempered to 45°C and poured in to sterile petri plates at the rate of 20 ml per plate, allowed to solidify and placed at ambient temperature for experimental use within 24 h of pouring the plates.

4.1.1.7.10 Nutrient agar – starch medium

This was prepared by boiling appropriate quantity of nutrient agar (4.1.1.7.7) containing soluble starch at 1% level, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity, plugged with cotton and autoclaved. The sterilized medium was tempered to 45°C and poured in to sterile petri plates at the rate of 20

ml per plate, allowed to solidify and placed at ambient temperature for use in experimental trials within 24 h of pouring the plates.

4.1.1.7.11 Simmons citrate agar

The medium composition is as follows (g/l):

Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0
Final pH 7.4 ± 0.2	

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in test tubes (18 x 150 mm) and autoclaved. After autoclaving, tubes containing simmons citrate agar were kept in a slanting position, so as to have 1" butt and remaining slants.

4.1.1.7.12 Sugar fermentation basal medium

The medium composition is as follows (g/l):

Peptone	10.0
Sodium chloride	5.0
Beef extract	3.0
Bromo cresol purple	0.04
Final pH 7.2 ± 0.2	

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in 3 ml amounts in the test tubes (12 x 75 mm) and autoclaved.

4.1.1.7.13 Sugar solutions

Requisite quantities of the stock solutions (10% each) of the following sugars were individually prepared in Milli Q water, membrane filtered (0.22 mμ filters) and stored in sterile screw-capped tubes at -20°C. The sugars used were glucose, mannitol and xylose.

4.1.1.7.14 Tributyrin agar

The medium composition is as follows (g/l):

Peptic digest of animal tissue	5.0
Yeast extract	3.0
Agar	15.0
Final pH 7.5 ± 0.2	

The requisite quantity of dehydrated medium was dissolved in water, added tributyrin supplement (FD081) (1 ml per 100 ml medium), mixed well and heated to boiling, sterilised by autoclaving and poured into sterile plates.

4.1.1.8 Molecular biology requisites

4.1.1.8.1 Ethidium Bromide stock solution (10 mg/ml)

Ethidium bromide in 10 mg of was dissolved in 1 ml of Milli Q water. The solution was dispensed in a microcentrifuge tube and stored in dark at 4 °C for further use.

4.1.1.8.2 Lysozyme

A 10 mg/ml stock solution of lysozyme was prepared in autoclaved Milli Q water and stored in aliquots at -20°C for use in experimental trials.

4.1.1.8.3 Proteinase K

A 20 µg/ml stock solution of lysozyme was prepared in autoclaved Milli Q water and stored in aliquots at -20°C.

4.1.1.8.4 Sodium acetate

A 3M solution of sodium acetate was prepared in Milli Q water, sterilized by autoclaving and stored at ambient temperature.

4.1.1.8.5 Sodium dodecyl sulphate (SDS) of 20%

A 20% stock solution of SDS was prepared in MIlli Q water, sterilized by autoclaving and stored at ambient temperature.

4.1.1.8.6 Sucrose solution

Requisite quantities of sucrose solution (6.7% w/v) was prepared in Milli Q water, membrane filtered (0.22 mµ filter) and stored in sterile screw-capped tubes at -20°C for further use in the experimental trials.

4.1.1.8.7 Tris acetate ethylene diamine tetra acetic acid (TAE) buffer (50 X)

Tris base of 24.2 g, 5.71 ml of glacial acetic acid and 10 ml of 0.5 M EDTA (pH 8.0) were added to 75 ml of Milli Q water. The pH of the buffer was adjusted to 7.2 and the final volume was made up to 100 ml with Milli Q water. The buffer was sterilized by autoclaving and stored at ambient temperature.

4.1.1.8.8 Tris ethylene diamine tetra acetic acid (TE) buffer (pH 8.0)

To 800 ml of Milli Q water, 10.0 ml of 1.0 M Tris of pH 8.0 and 2.0 ml 0.5 M EDTA of pH 8.0 were added and the total volume made up to 1000 ml with Milli Q water. If necessary, the pH was adjusted to 8.0 using HCl or NaOH. The solution was sterilized by autoclaving and stored at ambient temperature.

4.1.1.9 PCR primers

In the present study, selected toxigenic / pathogenic determinant genes in *B*. *cereus* were included to assess the prevalence of these traits in the native food isolates of *B. cereus*. The oligonucleotide primers for target genes were selected from available literature and got synthesized from a commercial company (Sigma Aldrich, Bangalore, India). The nucleotide sequences of the primers used in this study are shown in **Table 1**.

4.1.1.10 Primers for randomly amplified polymorphic DNA (RAPD)

Approximately 35 arbitrary primers for the RAPD reactions were initially screened for amplification against genomic DNA of *B*. *cereus* isolates. Based on good amplification pattern and reproducibility of initially screened primers, finally 4 primers designated as RA 08, RA 14, RA 18 and RA 26 were selected for the final experimental trials in RAPD-PCR. The nucleotide sequences of selected primers were as follows:

RA 08 – 5′ TGGCCGTGTG 3′

RA 14 – 5′ TTCGAGCCAG 3′

RA 18 – 5′ GATGACCGCC 3′

RA 26 – 5′ GGACACCACT 3’

All the oligonucleotide primers were decamers with 2 dinucleotide repeats each and synthesized from a commercial company (Sigma Aldrich, Bangalore, India).

Table 1 Nucleotide sequence of specific primers and PCR conditions used in the detection of *B. cereus*

Target gene	Primer designation	DNA sequences (5'-3') and PCR conditions	PCR product (bp)	References
16S rDNA	16S rDNA	GAGAGTTTGATCCTGGCTCAG	1468	Selvakumar et al. 2007
Universal eubacterial		CTACGGCTACCTGTTACGA		
		94°C 5′; 94°C 1′; 57°C 1′; 72°C 1′; 72°C 10′; (35 cycles)		
Phosphatidylinositol	Pi-PLC	AGTATGGGGAATGAC	342	Padmapriya et al. 2004
phospholipase C (*pi-plc*)		ACAATTTTCCCACGA		
NCBI Acc. No. M30809		94°C 5′; 94°C 1′; 50°C 1′; 72°C 1′; 72°C 8′; (35 cycles)		
Haemolysin BL (*hbl*)	Ha-1	TGCGAGGTGAAATTCAACAA	489	Radhika et al. 2002
NCBI Acc. No. L20441		GAACGCCCGAATATTGAGAA		
		94°C 5′; 94°C 1′; 50°C 1′; 72°C 1′; 72°C 8′; (35 cycles)		
Sphingomyelinase (*sph*)	Sph	CGTGCCGATTTAATTGGGGC	558	Hsieh et al. 1999
NCBI Acc. No. M20194		CAATGTTTTAAACATGGATGCG		
		94°C 5′; 94°C 20 s; 58°C 20s; 72°C 20s; 72°C 8'; (35 cycles)		
Cerulide synthetase (*ces*)	Ces	GACAAGAGAAATTTCTACGAGCAAGTACAAT	635	Ehling-Schulz et al. 2005
NCBI Acc. No. AY691650		GCAGCCTTCCAATTACTCCTTCTGCCACAGT		
		94°C 5′; 94°C 20 s; 60°C 30s; 72°C 20s; 72°C 8'; (35 cycles)		
Cytotoxin K (*cytk*)	Cytk	ACAGATATCGGTCAAAATGC	809	Guinebretiere et al. 2002
NCBI Acc. No. AJ277962		GAACTGCTAACTGGGTTGGA 94°C 5′; 94°C 20 s; 54°C 30s; 72°C 20s; 72°C 8'; (35 cycles)		

4.1.2 METHODOLOGY

4.1.2.1 Isolation of native food isolates of *B. cereus* by conventional selective plating protocols

Isolates of *B*. *cereus* were isolated from individual food samples by plating on a selective medium namely polymyxin egg yolk mannitol bromothymol blue agar (PEMBA). A homogeneous sample was prepared in 0.85% saline to give an initial 1:10 dilution. In case of liquid samples like raw milk, 11 ml of the sample was pipetted and mixed thoroughly with 99 ml of diluent. For solid samples, representative units of the sample weighing 11 g were blended with 99 ml diluent in a Stomacher. The isolation protocol is represented schematically as follows:

Requisite quantity of food samples were uniformly mixed with 99 ml of 0.85% saline under aseptic conditions to give an initial dilution of 1:10

↓

Homogeneous sample diluted appropriately with 0.85% saline

↓

Aliquots of 0.1 ml of requisite dilutions were surface plated on pre-poured PEMBA

↓

Inoculated plates were incubated for 24-48 h at 37°C

↓

Characteristic colonies of *B*. cereus were irregular shaped, peacock blue coloured with a halo zone of lecithinase activity around the individual colonies

Presumptive colonies of *B*. *cereus* were randomly selected based on characteristic colony features and purified by streaking on BHI agar plates. The individual purified colonies were selected and maintained at 4°C in the culture stock of this Department. Prior to use in experimental trials, the isolates were propagated successively twice in BHI broth for 24 h at 37°C in an Orbital Shaker Incubator (Alpha Labs., Bangalore, India) at 120 rpm.

4.1.2.2 Characterization of presumptive native food isolates of *B. cereus*

The presumptive native food isolates of *B. cereus* and the reference cultures of *B. cereus* were identified individually by morphological, cultural and biochemical characteristics according to the documented procedures (Cappucino and Sherman, 2004).

4.1.2.2.1 Gram's staining

The heat fixed smears of 18 h old broth culture of individual isolates of *B. cereus* prepared on a clean glass slide were stained with crystal violet for 1 min, followed by washing off excess stain with water. Then Lugol's iodine solution was added and allowed to react for 1 min. After washing off iodine with water, the smear was treated with 95% ethanol for 30 seconds, so as to remove the excess crystal violet. Finally, the smear was counter stained with safranin, allowed to react for 30 sec, washed with water, dried and examined under oil immersion of a compound microscope. Gram positive cells appear as violet coloured, while Gram negative cells as pink coloured. Besides, the cell morphology was also recorded.

4.1.2.2.2 Endospore staining

The heat fixed smears of 36-48 h old broth culture of individual isolates were prepared on clean glass slides. The slides were flooded with Malachite green and steamed for 5 min with the addition of stain at frequent intervals without allowing the slide to dry. Slides were then rinsed gently with water and counter stained with safranin for 30 seconds. The smear was washed with water, air-dried and examined under oil immersion of a compound microscope. The endospores get stained green and vegetative cells red. Vegetative cells appear rod shaped containing an elliptical spore located within the cell.

4.1.2.2.3 Delta endotoxin crystal staining

The heat fixed smear of 72 h old agar culture of individual isolates of *B. cereus* was stained with amido black for 10-15 min. The smear was rinsed with water and counter stained with carbol fuchsin for 10-15 seconds. The smear was rinsed with water, air-dried and examined

under oil immersion of a compound microscope. Endotoxin crystals, if present, would appear as characteristic black bipyramidal structures.

4.1.2.2.4 Catalase production

Test cultures were grown freshly on BHI agar slants for 20 h at 37°C, on to which were added few drops of 3% (v/v) hydrogen peroxide. Culture tubes were observed for the formation of nascent oxygen in the form of bubbles. This indicates positive for catalase production.

4.1.2.2.5 Test for motility and Oxidative / fermentative reaction

Stab inoculation of the test cultures individually were performed in the sterile tubes of Hugh Leifson medium. Each culture was inoculated in duplicate. To one set of the inoculated tubes, few drops of sterile liquid paraffin were added to overlay the agar medium (anaerobic condition). The other set of tubes were kept without any addition. Both the sets of tubes were incubated for 24-48 h at 37°C. Incubated tubes were observed for the spreading of the culture growth from the line of inoculation, which indicates the motile nature of the culture. Acid production from glucose was indicated by colour change of the medium from light purple to yellow. Positive tubes for acid production under aerobic condition indicate the oxidative nature, while positive tubes under anaerobic condition indicate fermentative nature.

4.1.2.2.6 Casein hydrolysis

A loopful of 20 h old broth culture of individual isolates was spot inoculated on pre-poured plates of nutrient agar-milk medium **(4.1.1.7.9)** by taking care to maintain appropriate space between individual spottings. Inoculated plates were incubated for 24-48 h at 37°C. A clear zone formed around the individual colonies is considered as positive reaction for casein hydrolysis.

4.1.2.2.7 Citrate utilization

Individual test cultures were inoculated into the prepared slants of simmons citrate agar (**4.1.1.7.11**) by making a stab in the butt and streaking on the slant. Inoculated tubes were incubated for 24-48 h at 28°C. Positive reaction for citrate utilization was observed by the development of deep blue colour in the slant.

4.1.2.2.8 Gas production from Glucose

MRVP medium **(4.1.1.7.5)** in 10 ml quantities was taken in test tubes of 18 x 150 mm and placed with inverted Durham tube, plugged with cotton, autoclaved and tempered to ambient temperature. The tempered tubes were inoculated with a loopful of 20 h old broth culture of individual isolates and incubated for 24-48 h at 37°C. Appearance of gas inside the Durham tube indicates the production of gas from glucose.

4.1.2.2.9 Gelatin hydrolysis

A loopful of 20 h old broth culture of individual isolates was spot inoculated on pre-poured plates of nutrient agar-gelatin medium **(4.1.1.7.8)** by taking care to maintain appropriate space between individual spottings. Inoculated plates were incubated for 24-48 h at 37°C. Following incubation, plates were flooded with $HgCl_2$ solution and observed for a clear zone formed around the individual colonies, which was considered as positive reaction for gelatin hydrolysis.

4.1.2.2.10 Growth at 6, 10, 50 and 65 °C

A loopful of 20 h old broth culture of individual isolates was inoculated in 4 sets of nutrient broth **(4.1.1.7.7)** tubes. Two individual sets were incubated respectively at 6 and 10 °C for 8-10 d, while the other two sets of tubes were incubated at 50 and 65 °C, respectively, for 24-36 h. Appearance of turbidity in the incubated broth tubes was taken as indication of positive growth of the inoculated isolates.

4.1.2.2.11 Growth in 7% sodium chloride

Nutrient broth tubes **(4.1.1.7.7)** were prepared with sodium chloride at a concentration of 7% and inoculated with a loopful of 20 h old broth culture of individual isolates and incubated for 24-48 h at 37°C. Appearance of turbidity in the incubated broth tubes was taken as indication of positive growth of the inoculated isolates at 7% salt concentration.

4.1.2.2.12 Haemolysis in blood agar

Cell free culture supernatants of individual test cultures grown for 6 h at 30°C in 50 µl aliquots were added in to 5 mm diameter wells made in pre-poured blood agar plates **(4.1.1.7.2)**. The plates were incubated at 37°C and observed at frequent time intervals under bright light for the formation of a discontinuous pattern of haemolysis surrounding the well during the initial hours of incubation, followed by the clear zone of haemolysis upon extended incubation indicating beta haemolysis.

4.1.2.2.13 Lipase activity

A loopful of 20 h old broth culture of individual isolates was spot inoculated on pre-poured plates of 1% Tributyrin agar **(4.1.1.7.14)** by taking care to maintain appropriate space between individual spottings. Inoculated plates were incubated for 24-48 h at 37°C. Appearance of a transparent zone around the colony is an indication of lipase activity by the isolates.

4.1.2.2.14 Methyl red and Voges Proskauer (MR-VP) reaction

These two tests were performed using MR-VP broth (**4.1.1.7.5**). Test cultures individually were inoculated into the broth medium and incubated for 24 h at 37°C. The culture broth was divided into two parts, one part was used for MR reaction and the other for VP reaction.

Methyl red test:

Methyl red indicator was prepared by dissolving 0.1 g methyl red in 300 ml of 95% ethanol and later made upto 500 ml with distilled water. Add 5-6 drops of MR reagent to one part of the culture broth. Development of pink colour indicates positive reaction.

Voges Proskauer (VP) test:

VP test reagent consists of 2 solutions

Solution A		**Solution B**	
α-napthol	5 g	Potassium hydroxide	40 g
Absolute alcohol	95 ml	Creatine	0.5 g
		Distilled water	100 ml

To the second part of culture broth, 0.6 ml of solution A and 0.2 ml of solution B were added, mixed well and tubes kept unplugged so as to allow for the incorporation of air. Formation of eosin pink colour indicated positive reaction.

4.1.2.2.15 Nitrate reduction

Individual test cultures were inoculated into prepared nitrate broth tubes (**4.1.1.7.6**) and incubated for 24 h at 37°C. The culture broth was tested for nitrate reduction using the following reagent, which consisted of two solutions:

Solution 1		**Solution 2**	
Sulphanilic acid	8 g	α-napthol	5 g
5N Acetic acid	1000 ml	5N Acetic acid	1000 ml

To 5 ml of the 24 h old culture broth was added 2 drops each of solution (1) and (2). The development of orange/brick red colour was indicative of nitrate reduction to nitrite.

4.1.2.2.16 Starch hydrolysis

A loopful of 20 h old broth culture of individual isolates was spot inoculated on pre-poured plates of nutrient agar-starch medium **(4.1.1.7.10)** by taking care to maintain appropriate space between individual spottings. Inoculated plates were incubated for 24-48 h at 37°C. Following incubation, plates were exposed in a glass chamber saturated with iodine vapours,

to observe for the development of dark blue colour around the individual spot as an indication of positive reaction for starch hydrolysis.

4.1.2.2.17 Acid production from sugars

To a set of 2 tubes of 3 ml each of sugar fermentation basal medium (**4.1.1.7.12**) was added 0.3 ml each of the 10% membrane filtered individual sugar solutions of mannitol and xylose (**4.1.1.7.13**). These tubes were then inoculated with the individual test cultures, mixed well and incubated at 37°C for a period of 5 d. Incubated tubes were observed at 24 h intervals for acid production by the colour change in the medium i.e. from purple to yellow.

4.1.2.3 PCR detection of selected potent toxigenic traits among the identified native food isolates of *B. cereus*

4.1.2.3.1 Isolation of DNA from identified isolates of B. cereus

The identified isolates of *B. cereus* and the reference cultures were grown individually in BHI broth for 14 h at 37°C in an orbital shaker incubator (Alpha Labs., Bangalore, India) at 140 rpm. Aliquots of culture broths were plated on BHI agar to obtain discrete colonies. Single and well-isolated colonies were once again grown in BHI broth for 14 h at 37°C at 140 rpm. The total genomic DNA of individual isolates was extracted by phenol-chloroform method as described by Schraft and Griffiths (1995). The protocol was as follows:

- Cells from 1.5 ml of 14 h old culture broth was harvested by centrifugation at 10000 rpm for 10 min at 4^{o}C
- The supernatant was discarded and to the resulting pellet was resuspended in 0.85% sterile saline
- The suspension was centrifuged at 8000 rpm for 10 min at 4°C
- The supernatant was discarded and the pellet was suspended in 400 µl sucrose solution (6.7% w/v), 25 µl of lysozyme (10 mg/ml) and incubated for 60 min at 37°C
- After incubation, 50 µl of 20% (w/v) SDS was added and incubated for 30 min at ambient temperature, followed by addition of 5 µl of proteinase K solution (20 µg/ml) and placed for 30 min at 37°C
- Equal volume of phenol (equilibrated) was added, gently inverted to mix and centrifuged at 8000 rpm at 4°C for 10 min

- The resulting supernatant was transferred to fresh microcentrifuge tube and equal volume of chloroform added and gently inverted to mix and centrifuged at 8000 rpm at 4°C for 10 min
- The resulting supernatant was transferred to fresh microcentrifuge tube, to which were added 50 µl of 3 M sodium acetate and 2 ml of chilled ethanol and then placed overnight for cold precipitation at -20°C
- The precipitated mixture was centrifuged at 5000 rpm at 4°C for 10 min
- To the resulting cell pellet, 2 ml of 70% ethanol was added and mixed by gentle inverting
- The prepared suspension was centrifuged at 8000 rpm at 4°C for 10 min, after which the supernatant was discarded and the resultant pellet was air dried for 1-2 h
- The dried pellet (template DNA) was then resuspended in 20 µl TE buffer and stored at -20°C until further use in the experiment trials

4.1.2.3.2 PCR amplification for species (B. cereus) specific target gene

Identified isolates of *B. cereus* were subjected to uniplex PCR with species specific primers (Pi-PLC) of phosphatidylinositol phospholipase C gene (*pi-plc*) as detailed in **Table 1**. The Reaction components for PCR amplification included the following:

i. Template DNA
ii. Gene specific primers (**Table 1**)
iii. *Taq* DNA polymerase (3 units/µl, Bangalore Genei, India)
iv. 10X Reaction Buffer: 100 mM Tris pH 9.0, 500 mM KCl, 15 mM $MgCl_2$ & 0.1% Gelatin
v. Nuclease-free water
vi. dNTP mix (10mM of each dNTP)

For a reaction volume of 25 µl, the following reaction components were combined in a thin-walled 0.2 ml PCR reaction tube to make a final volume of 25 µl.

Components	Volume (µl)	Final concentration
Nuclease-free water	16.7	
10 X Reaction Buffer*	2.5	1 X
dNTP mix, 10 mM (2.5 mM each)	0.5	0.2 mM
Taq DNA polymerase (3U/ µl)	0.3	0.03 U/µl
Gene specific Primer F	0.5	0.2 µM
Gene specific Primer R	0.5	0.2 µM
Template	4.0	

* The reaction buffer contained 15 mM $MgCl_2$. The final concentration of $MgCl_2$ in the reaction was 1.5 mM

The contents of the tubes were mixed by a brief spin in a microcentrifuge. PCR amplification was performed in an automated DNA thermal Cycler (Eppendorf, Master Cycler, Cedex, France) following the PCR conditions as detailed in **Table 1**.

4.1.2.3.3 PCR amplification for target genes (including toxigenic traits)

The native isolates of *B*. *cereus* positive for pi-plc gene were evaluated for the prevalence of universal eubacterial 16S rDNA and selected toxigenic target genes like haemolysin (*hbl*), sphingomyelinase (*sph*), cytotoxin K (*cytk*) and cerulide synthase (*ces*) genes. This was undertaken by subjecting these positive isolates to uniplex PCR amplification with primers of 16S rDNA, Ha-1, Sph, CytK and Ces under the defined respective PCR conditions as detailed in **Table 1**. The PCR protocols for amplification of these primers were the same as described under **4.1.2.3.2**.

4.1.2.3.4 Duplex PCR amplification for pi-plc and hbl target genes

Duplex PCR to detect two target genes namely *pi-plc* and *hbl* in confirmed isolates of *B*. *cereus* was undertaken by subjecting to PCR using both Pi-PLC and Ha-1 primers as detailed in **Table 1**. In this case also, for a reaction volume of 25 μl, the following were the reaction components in a final volume of 25 μl.

Components	Volume (μl)	Final concentration
Nuclease-free water	15.7	
10 X Reaction Buffer*	2.5	1 X
dNTP mix, 10 mM (2.5 mM each)	0.5	0.2 mM
Taq DNA polymerase (3U/ μl)	0.3	0.03 U/μl
Gene specific Primer A (Forward)	0.5	0.2 μM
Gene specific Primer A (Reverse)	0.5	0.2 μM
Gene specific Primer B (Forward)	0.5	0.2 μM
Gene specific Primer B (Reverse)	0.5	0.2 μM
Template	4.0	

* The reaction buffer contained 15 mM $MgCl_2$. The final concentration of $MgCl_2$ in the reaction was 1.5 mM

The contents of the tubes were mixed by a brief spin in a microcentrifuge. PCR amplification was performed in an automated DNA thermal Cycler (Eppendorf, Master Cycler, Cedex, France) following the PCR conditions as detailed in **Table 1**.

4.1.2.3.5 Analysis of PCR amplified products by agarose gel electrophoresis

The resultant PCR amplified products were analyzed by agarose gel electrophoresis following the method described by Sambrook and Russel (2001).

Requisites

i. Agarose
ii. Tris-acetic acid-EDTA (TAE) buffer (50 X) – as described under **4.1.1.8.7**
iii. Orange loading dye (6X)
iv. Gel casting tray
v. Submarine gel electrophoretic apparatus and power supply
vi. Ethidium bromide stock solution (10 mg/ ml) – as described under **4.1.1.8.1**

The electrophoresis was performed as follows:

i. The gel casting tray was assembled and the comb was installed at the appropriate position for the wells.
ii. Agarose in 2 g quantity was added to 100 ml of 1 X TAE buffer. The mixture was boiled to dissolve agarose.
iii. The solution was cooled to 50°C and poured into the gel casting tray.
iv. The gel was allowed to set. The comb was removed and the gel was placed in the electrophoresis tank.
v. Sufficient volume of 1X TAE buffer was added to the tank to cover the surface of the gel.
vi. A 10 μl aliquot of each sample was loaded after mixing with 2 μl of the loading dye.

vii. A molecular size marker prepared according to the manufacturer's (Bangalore Genei, Bangalore, India) instructions was loaded simultaneous with the samples in to a separate well.

viii. Electrophoresis was carried out at 100 volts until the dye reached 3/4th of the gel.

ix. The gel was removed from the tank and stained by soaking in a solution of 0.5 μg/ml ethidium bromide for 30 min at ambient temperature

x. The gel was destained in distilled water for 10 min, placed on a UV-transilluminator and the image was documented in Gel Documentation System (Vilber Lourmat, France).

4.1.2.4 Molecular basis for relatedness / diversity of selected isolates of *B. cereus*

Considering the positive results with PCR primers and activities of haemolysis, four native isolates of *B. cereus* namely CFR 1529, 1530, 1534 and 1536 were subjected for PCR amplification with the respective 16S rDNA, Pi-PLC, Ha-1 and Sph primers. The resultant PCR amplified products were purified using commercially available PCR purification Spin Kit (HiPur A, HiMedia Laboratories, Mumbai, India) and subjected to sequence analysis (Chromous Biotech., Bangalore, India). The partial nucleotide sequences of these specific target genes were subjected to BLAST programme of NCBI (Altschul et al., 1997) to assess the per cent homology with closely related strains and species of *B. cereus* cluster documented in Gene Bank database. A similarity network tree was constructed based on Force Type of Neighbour-Joining method from the same programme. Multiple alignment sequences was performed using partial nucleotide sequences obtained for the respective toxigenic target genes in the four native food isolates of *B. cereus* against selected strains of *B. cereus* / *B. anthracis* / *B. mycoides* / *B. thuringiensis* showing high degree of homology using Multalin version 5.4.1 (Corpet, 1988).

4.1.2.5 Clustering patterns among potential toxigenic native food isolates of *B. cereus* by RAPD-PCR

4.1.2.5.1 Bacterial cultures

This experimental trial included 12 potent toxigenic native food isolates of *B. cereus* - CFR 1506, 1508, 1521, 1525, 1529, 1530, 1532, 1533, 1534, 1535, 1536 and 1540, which were maintained in the culture stock of the Department of Human Resource Development, where the study was undertaken. The cultures were individually maintained at 6°C on Brain Heart Infusion (BHI) agar slants and propagated in BHI broth at 37°C, prior to use in experiments.

4.1.2.5.2 RAPD-PCR analysis

The total genomic DNA of individual isolates of *B. cereus* was extracted from 1.5 ml aliquots of BHI culture broth by Phenol-chloroform method and ethanol precipitation as previously described under **4.1.2.3.2**. The dried DNA was then resuspended in TE buffer of pH 8.0 and determined for quality and quantity at 260 nm in a UV-VIS Spectrophotometer (Genesys 5, Milton Roy Co., USA). This DNA was used as template for amplification of random primers in RAPD-PCR as described under **4.1.1.10**.

The protocol for RAPD-PCR was in accordance with the documented procedure of Williams et al. (1990). Amplification of DNA was performed in a total reaction volume of 25 µl, which consisted of 2.5 µl of assay buffer (10 mM Tris-HCl of pH 9, 1.5 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin of 10X concentration), 1 U of *Taq* DNA polymerase, 0.2mM dNTPs (Bangalore Genei, Bangalore, India), 2 µl of primer (0.2 µM) and 4 µl (~ 25-50 ng) of template DNA and 16.7 µl of Milli Q water. The thermocycling programme for the RAPD reaction was an initial denaturation under conditions of 94°C for 4 min, followed by 30 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 1 min and final extension at 72°C for 5 min performed in an automated DNA Thermal Cycler as described previously. The PCR amplified products in aliquots of 15 µl were run along with 100 bp DNA ladder in 2% agarose gels in 1X Tris acetate buffer for 2 h at 100 V and stained in 0.5 µg/ml ethidium bromide solution, followed by documentation in Gel Documentation System as described previously.

4.1.2.5.3 RAPD data analysis

The well-resolved fragments in RAPD profiles, ranging from 100-1000 bp were scored as present (1) or absent (0) for each analysis. Bands with the same migration distance were considered homologous. By taking into consideration the total number of distinct DNA-amplified fragments corresponding to different sizes, a pair-wise similarity matrix was computed and analyzed with Numerical Taxonomy System (NTSYS) version 2.02 (Rohlf, 1998) using the simple matching coefficient (Sokal and Michener, 1958). The similarity matrix was used to construct a dendrogram by the unweighed pair-group method with arithmetical averages (UPGMA) to determine the genetic relatedness between the isolates.

4.1.3 RESULTS AND DISCUSSION

4.1.3.1 Prevalence and characterization of food isolates of *B. cereus*

The viable populations of *B*. *cereus* from a diverse range of foods were enumerated by conventional selective plating using PEMBA medium (**Figure 1**). The mean viable counts ranged from a minimum of 3.4 $\log_{10}$ CFU/g in case of milk based foods to a maximum of 5.1 $\log_{10}$ CFU/g in spice based traditional fast foods. It was of relevance to present the incidence pattern of only *B*. *cereus* cultures against the prevailing background flora of non-*B*. *cereus* cultures in different types of traditional fast foods. The per cent incidence of *B. cereus* isolates in relation to samples analyzed range from a highest of 40% in milk based foods to a lowest of 10% in samples of processed wheat based foods.

Morphological, cultural and biochemical characterization of food isolates revealed that from a total of 65 food samples, 26 isolates were identified to belong to *B*. *cereus* cluster, which exhibited distinct features of typical *B*. *cereus* cluster (**Table 2**). As could be seen from the same table, the positive reactions exhibited by these isolates to a good number of characteristics give an indication of the versatility being inherent to *B*. *cereus*. The ability of isolates of *B*. *cereus* to grow in the range of 6 to 50°C adds to the versatility and definitely makes them to be appropriately called as opportunistic pathogen. Further, these native isolates also were positive for major enzymatic activities like hydrolysis of starch,

casein and gelatin. In contrast to this, Oguntoyinbo and Oni (2004) in their studies reported a very poor enzymatic potential of the isolates of *B*. *cereus*. The viable populations and distribution pattern of characterized isolates of *B*. *cereus* in foods are presented in **Table 3**.

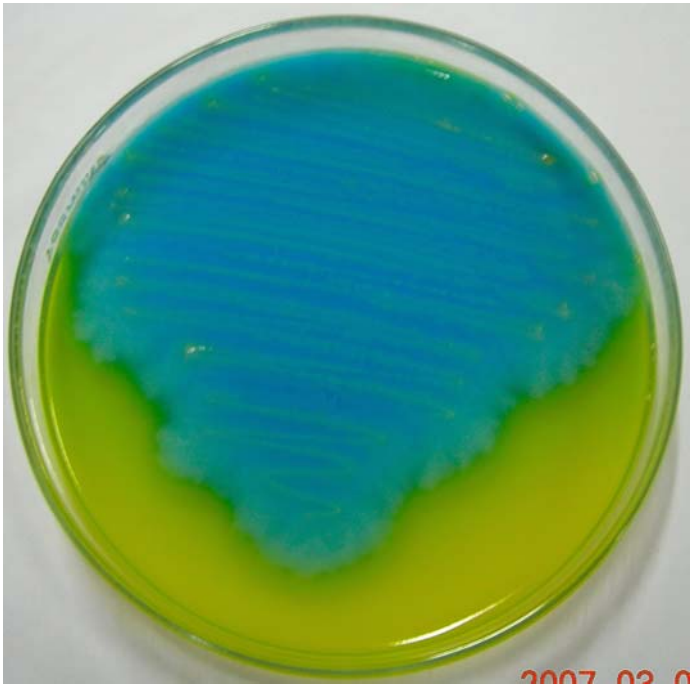


Figure 1 Native isolate of *B*. *cereus* streaked on to selective plate of PEMBA

Table 2 Characterization of presumptive native isolates of *Bacillus* spp.

Isolate No.	Characteristics											
	Growth at 6°C	Growth at 10°C	Growth in 7% NaCl	NO_3 Redn.	Hydrolysis of Starch	Hydrolysis of Gelatin	Hydrolysis of Casein	Acid from Mannitol	Acid from Xylose	MR Reaction	VP Reaction	Identified species
BC 05	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1505
BC 06	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1506
BC 07	–	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1507
BC 08	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1508
BC 09	+	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1509
BC 10	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1510
BC 15	–	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1515
BC 17	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1517
BC 21	–	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1521
BC 22	+	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1522
BC 24	–	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1524
BC 25	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1525
BC 26	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1526
BC 27	–	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1529
BC 28	–	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1530
BC 29	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1531
BC 30	–	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1532
BC 31	–	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1533
BC 32	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1534
BC 33	–	–	–	+	+	+	+	–	–	–	+	*B. cereus* CFR 1535
BC 34	–	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1536
BC 35	–	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1537
BC 36	–	–	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1538
BC 37	–	–	–	+	+	+	+	–	–	–	+	*B. cereus* CFR 1539
BC 38	–	+	–	+	+	+	+	–	–	–	+	*B. cereus* CFR 1540
BC 39	+	+	+	+	+	+	+	–	–	–	+	*B. cereus* CFR 1541

All the isolates were **positive** for Gram's & endospore staining, motility, growth at 50°C, growth under aerobic & anaerobic conditions and catalase; and n**egative** for endotoxin crystal staining, gas from glucose, mannitol and xylose utilization and growth at 65°C
CFR, represents the abbreviation for the host institute

Table 3 Viable counts and distribution pattern of isolates of *B. cereus* in foods

Food sample	*Bacillus cereus*				
	No of samples analyzed (n)	Samples positive for *B. cereus*		Viable count (log_{10} CFU/g)	Characterized isolates[a]
		(n)	(%)	mean ± SD	
Processed rice based foods added with spices and vegetable salads (Traditional fast foods)	30	05	16.6	5.1 ± 1.3	**5**/CFR[b] 1508, 1521, 1525, 1532, 1536
Milk and milk based foods	10	04	40.0	3.4 ± 0.2	**4**/CFR 1533, 1534, 1535, 1540
Processed wheat based foods	10	01	10.0	4.3 ± 1.5	**1**/CFR 1506
Spiced cooked rice based foods	15	02	13.0	4.5 ± 0.7	**2**/CFR 1529, 1530

[a] Characterized isolates were representatives of individual sample types under the listed categories

[b] CFR, represents the abbreviation for the host institute

The public health significance of *B*. *cereus* is of high concern in view of this organism being implicated in a large number of food poisoning outbreaks, world wide. Studies on the prevalence of *B. cereus* in different foods undertaken by researchers of other countries and India present a scenario similar to the one observed in the present study. A similar level of prevalence (40-45%) as observed in this study was reported by Smith et al. (2004), Reyes et al. (2007) and Altayar and Sutherland (2006) in retail chicken products, dried milk products and raw & processed vegetables, respectively. Lurlina et al. (2006) reported an incidence of 50% of *B. cereus* in samples of Port Salut Argentina cheese along with the presence of either *B. subtilis* and/or *B. licheniformis*. Similarly, an incidence of 40% of *B. cereus* in open samples of ice cream was recorded as against 26% in packed samples (Warke et al. 2000). This indicates the post-processing contamination of foods by the organism which may eventually lead to public health hazards.

In the Indian context, an incidence of 40-54% was reported in milk and milk products (*burfi, rasgolla, rasmalai and khoa*) with mean viable counts ranging from 3 $\log_{10}$ to 6 $\log_{10}$ CFU/g (Bedi et al. 2005; Hussain et al. 2005). In an earlier study, Shah et al. (1996) reported the prevalence in almost all the food samples studied by them and the incidence was higher in rice-based products.

Shankar et al. (1994) isolated and characterized the presence of psychrotrophs in ice cream samples with viable count ranging from 2 $\log_{10}$ to 3 $\log_{10}$ CFU/g. Similarly, Rama Raju et al. (1989) reported psychrotrophic strains of *B*. *cereus* from samples of cow and buffalo milk. This assumes significance in the backdrop of the incidence of psychotropic isolates *B. cereus* (which could grow at 6ºC) in the present work. Prevalence and emergence of psychrotrophic isolates may pose to be a cause of concern owing to the increased trend of storing foods under refrigerated conditions and consumption of frozen foods.

Pillai et al. (1993) reported 21-33% incidence of *B. cereus* strains from commercial dairy products like *lassi*, butter and ice cream sold by local vendors in the

City of Madras (now Chennai) in southern India, which was almost comparable with the prevalence level observed in milk and milk products (**Table 3**) of this study. The isolation of *B. cereus* from a diverse range of foods indicates the versatility of this pathogen. Similarly, strains of *B. cereus* have been isolated from a variety of sources like pulses, rice and rice products, oils, fish, meat, spices and milk & milk products (Kamat et al. 1989; Shah et al. 1996).

Despite product profile, which could be unfavourable for survival and multiplication of this organism, in the present study, the occurrence of *B. cereus* in milk and rice-based foods may be the result of prevailing environmental conditions combined with post-processing practices. In general, the isolates of *B. cereus* tend to behave as mesophilic. However, changes in environmental conditions could bring in the predominance of psychrotrophic strains of *B. cereus* (Warke et al. 2000; Valero et al. 2007).

4.1.3.2 Identity of *B. cereus* and determination of associated toxigenic traits in native isolates by PCR detection

Through the use of Pi-PLC primers (**Table 1**) designed specifically to detect *B. cereus* isolates, 12 isolates (46%) of this cluster gave a positive amplification in PCR. The focus of present study was to detect food isolates of *B. cereus* (Pi-PLC confirmed), which could harbour potent toxigenic factors. The pattern of characterized *B. cereus* isolates exhibiting positive PCR reactions and haemolytic activity are presented in **Table 4**. All the 12 isolates showed positive amplification with 16S rDNA and Pi-PLC primers in PCR, which confirmed the species of *B. cereus*. Among these 12 isolates of *B. cereus*, 8 (67%) and 6 (50%) isolates showed positive amplification of haemolysin BL (*hbl*) and sphingomyelinase (*sph*) genes, respectively, by uniplex PCR. Besides, the use of duplex PCR with Pi-PLC and Ha-1 primers showed positive amplification in 8 (67%) isolates, which were positive with these respective primers in uniplex PCR (**Table 4**). A representative pattern of duplex

(Pi-PLC and Ha-1) and uniplex (Sph and 16S rDNA, respectively) PCR in selected four isolates of *B. cereus* is shown in **Figure 2**. Amongst isolates tested for haemolysin BL activity (**Table 4**), 67% (8 out of 12 isolates) exhibited discontinuous haemolytic pattern on blood agar (**Figure 3**). However, none of these isolates showed any positive amplification with primers of cerulide (Ces) and cytotoxin K (Cytk).

Table 4 Distribution pattern of *B. cereus* isolates for reactions with primers for toxigenic traits in PCR and haemolytic activity by plate assay

B. cereus isolates[a]	PCR reactions[a]			Haemolytic activity
	Uniplex		Duplex (Pi-PLC and Ha-1)	Discontinuous pattern
	Ha-1	Sph		
CFR 1506	+	-	+	-
CFR 1508	+	-	+	-
CFR 1521	+	-	+	+
CFR 1525	+	-	+	+
CFR 1529	+	+	+	-
CFR 1530	+	+	+	-
CFR 1532	-	+	-	+
CFR 1533	-	-	-	+
CFR 1534	+	+	+	+
CFR 1535	-	+	-	+
CFR 1536	+	+	+	+
CFR 1540	-	-	-	+

[a]All the native isolates were positive with 16S rDNA and Pi-PLC primers

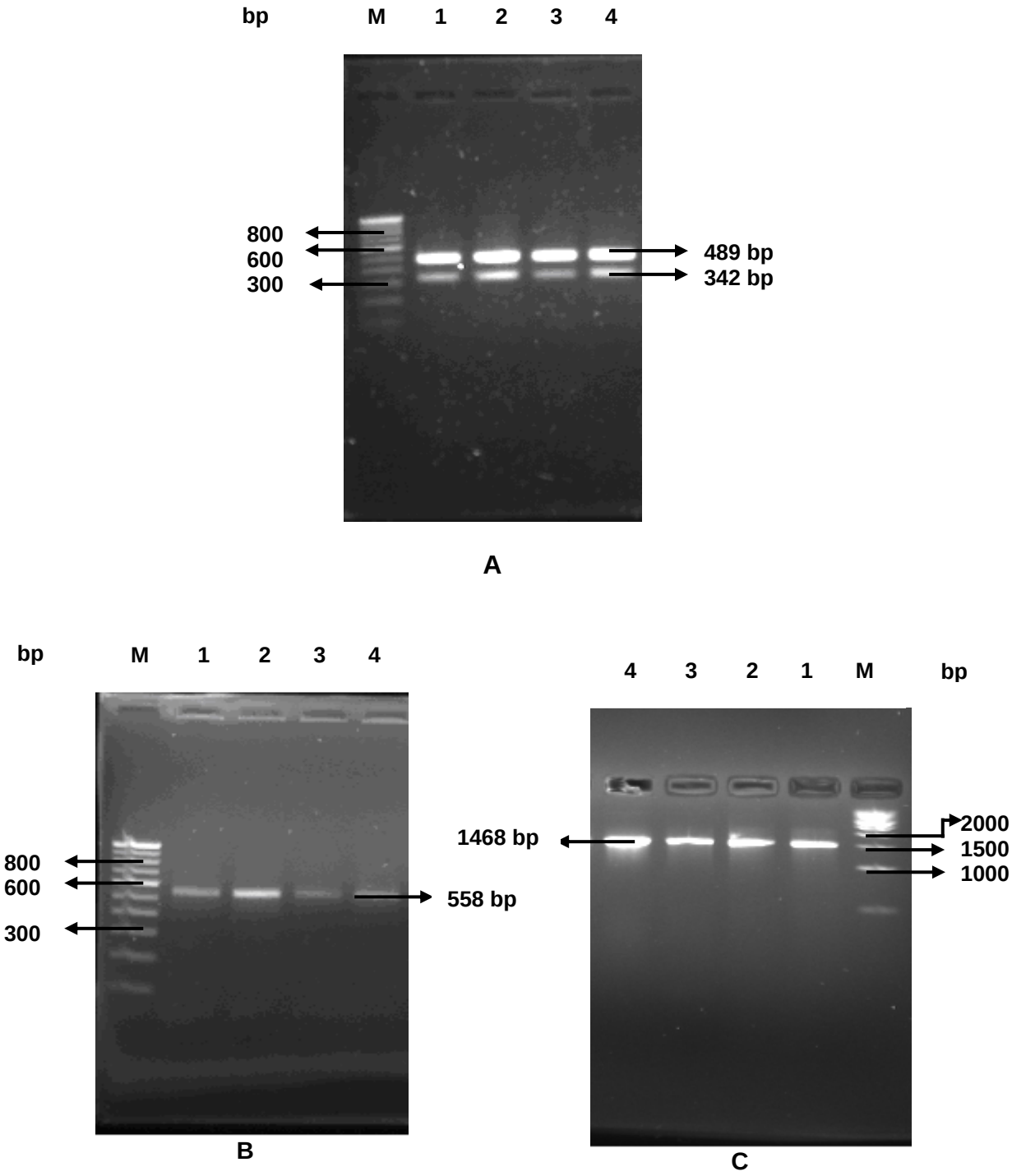


Figure 2 Agarose gel electrophoretic pattern of PCR products showing amplicons with Pi-PLC and Ha-1 primers in duplex [**A**] and uniplex with Sph [**B**] and 16S rDNA [**C**] primers in cultures of *B. cereus*; Lane M, 100-1000 bp marker in [**A**] and [**B**]; Lane M, 500-5000 bp marker in [**C**]; Lanes 1-4, Native isolates of *B. cereus* CFR 1529, CFR 1530, CFR 1534 and CFR 1536

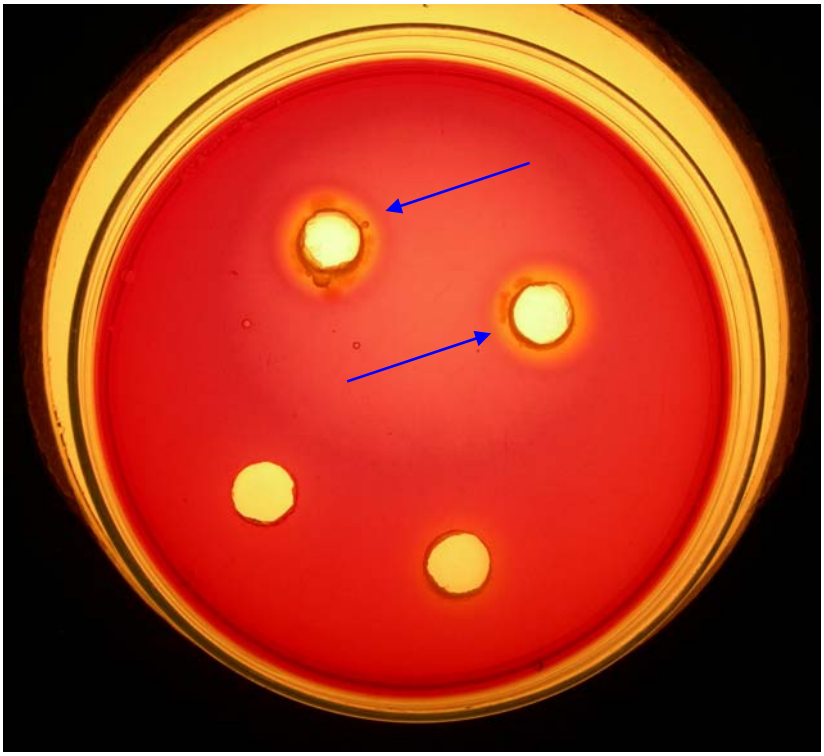


Figure 3 Haemolytic activity (Discontinuous haemolysis) among native food isolates of *B. cereus*

DNA based tests like PCR are proven to be highly sensitive and specific with several advantages for the detection of the organisms. Furthermore, these methods can be made more specific by using appropriate species-specific primers targeting unique sequences. The versatility of the technique makes it useful for direct detection of the organism without pre-enrichment and for such organisms which cannot be cultured or difficult to culture by routine laboratory procedures. In addition to the species-specific detection of *B. cereus*, phosphatidyl inisitol specific phospholipase C is considered as a virulence factor, since it is understood to cause degradation of cell and mucous membranes, which are rich in phospholipids, leading to necrosis (Gilmore et al. 1989). Schraft and Griffiths (1995) developed primers for detection of lecithinase positive *Bacillus* species, which in combination with PCR hybridization

assay could detect *B. cereus* up to the limit of 1 CFU/ml of milk. Similarly, Radhika et al. (2002) detected *B. cereus* by colony hybridization using the PCR-generated probes of phospholipase. Manzano et al. (2003b) detected *B. cereus* strains from a coffee concentrate sample used in industrial preparation by molecular based approach.

The 16S rDNA sequences are known to be highly conserved during the evolution and therefore used extensively for taxonomic identification and further confirmation of the isolates to their species level. Nakano et al. (2004a) detected *B. cereus* by spiking foods like milk, butter, cream etc. after a brief enrichment. Priha et al. (2004) designed a semi-quantitative method of detection of *B. cereus* group cells based on real time amplification of 16S rDNA sequence on cardboard and paper samples, resulting in a detection level of 2 $\log_{10}$ CFU/g. Selvakumar et al. (2007) amplified the 16S rDNA sequences by using the universal eubacterial primers for successful identification and confirmation of *B. cereus*. The amplification of the 16S rDNA sequences for the taxonomic identification of the strains used in the study assumes significance in the light of *B. cereus* being closely related to the other members of the *B. cereus* group. In addition to the 16S rDNA sequences, Bavykin et al. (2004) demonstrated that use of 23S rDNA and the *gyr* sequences could be successfully used for the differentiation of *B. anthracis* strains from the rest of the *B. cereus* group members.

Although diarrhoeal food poisoning arising out of *B. cereus* is due to 3 enterotoxins namely haemolysin BL (*hbl*), non-haemolytic enterotoxin (*nhe*) and cytotoxin K (*cytk*), the major health hazard attributed to *B. cereus* is mostly related with *hbl* enterotoxin complex (Hsieh et al. 1999). The diarrhoeal enterotoxin, haemolysin BL was first isolated by Beecher and MacMillan (1990). The toxin is known to be produced in the small intestine of the host. The time needed for the onset of illness is 8-16 h and the duration of the illness is 12-24 h. The toxin causes abdominal pain, watery diarrhoea and malaise. The toxin consists of 3 components

namely B, L_1 and L_2 and all the 3 are required for biological activity. It produces discontinuous pattern of haemolysis on sheep blood agar plates (Beecher and Wong, 1997).

Among the 12 isolates tested positive for Pi-PLC primers, 8 (67%) were found to exhibit haemolytic activity. A definite relationship could not be arrived between the presence of *hbl* gene and assay for haemolytic activity in blood agar plates. A similar observation has been reported in an earlier study, in which despite the presence of haemolysin BL gene, haemolytic activity was not observed which was attributed to the designing of primers which were highly strain specific (in't Veld et al. 2001). The enterotoxigenicity among the isolates of *B. cereus* has been established to the presence of all 3 components namely a binding component B and lytic components L_1 and L_2 in *hbl* complex (Schoeni and Wong, 2005). In the present study, the Oligonucleotide primers were targeted for the B component of haemolysin BL gene complex. Hence, it is likely that among the PCR positive isolates of *B. cereus*, there may be the absence of either one or both lytic components (L_1 and $L_{2)}$) of the haemolysin BL gene complex (Radhika et al. 2002).

DNA-based molecular approaches for the detection of haemolysin BL enterotoxin targeting the components of gene has been carried out by several research investigators. Oltuszak-Walczak (2006) detected haemolytic strains of *B. cereus* from food and environmental samples. Similarly, Suwicha-Thaenthanee et al. (2005) detected and compared the phenotype and genotype profiles of *B. cereus* isolated in Thailand. In a similar line of work, cake samples implicated in food poisoning outbreaks, wherein several people took to illness in Pisa, Italy were subjected for the detection of different toxigenic traits like *pi-plc*, *sph*, *cytk*, *hbl*, and *ces* (Ghelardi et al. 2002). Like-wise, screening for the presence of haemolysin BL and cytotoxin K genes resulted in the detection of enterotoxigenic strains of *B. cereus* in samples of selected retail chicken products (Smith et al. 2004).

It is concluded from an earlier study that phospholipase C and sphingomyelinase are encoded by two tandemly arranged genes with close genetic linkage, which constitute a biologically functional cytolytic determinant and act in natural concert causing lysis of target cells and thus regarded as an effective cytolysin (Gilmore et al. 1989; Beecher and Wong, 2000). Studies have revealed that the potential virulence factors (haemolysin, sphingomyelinase and phospholipase C) are under the control of pleitropic regulator *plc R* and are expressed among these virulence factors, which may be co-operative, synergistic and antagonistic (Agaisse et al. 1999 and Slamti et al. 2004).

Cerulide toxin is known to cause emesis characterized by nausea and vomiting. It is stable to heat and pH and is found to be more associated with farinaceous foods like rice and rice products. Cytotoxin K is a single component haemolytic protein causing necrotic enteritis outbreaks (Lund et al. 2000). In the present study, PCR detections using Ces and Cytk primers were performed to detect for the presence of corresponding genes. However, none of the native isolates exhibited amplification in PCR for these two toxigenic traits.

The enterotoxigenic strains of *B. cereus* from a diverse range of food products were isolated by earlier researchers. Reyes et al. (2007) recovered both, psychotropic and enterotoxigenic strains of *B. cereus* from several dried milk products in Chilean school feeding programme. Larsen and Jogersen (1997) isolated several psychrotrophic strains of *B. cereus* from pasteurized full fat and low fat milk in Denmark with the viable counts ranging from 3 $\log_{10}$ CFU/ml to 5 $\log_{10}$ CFU/ml. Studies from Sutherland (1993) showed that spores of a toxigenic and psychrotrophic dairy isolate of *B. cereus* (HRM 44) were unable to grow and produce diarrhoeagenic toxin at 6ºC in creams and dairy products and growth could be demonstrated when stored at 21ºC. However, the potential danger caused by the germinating spores still continues in the wake of the non-availability of efficient cold chain facility at all places. In contrasting studies, Choma et al. (2000) reported strains of *B. cereus* in

cooked-chilled and pasteurized vegetable products which were enterotoxigenic, while Oguntoyinbo and Oni (2004) reported enterotoxigenic strains from vegetable proteins in seasonings in Nigeria. Gasaluck et al. (1996) and Kim et al. (2004) reported strains from cereal-legume based products such as *kapi nam-pla* and raw soy bean sprouts, respectively. Rusul and Yacoob (1995) reported diarrhoeal strains from cereal products like rice noodles, wheat noodles and spices. Also several strains were recovered from meat and meat products consumed in Turkey, which were present in levels high enough to cause public health hazard (Guven et al. 2006).

In the Indian context, several studies have reported the incidence of enterotoxic *B. cereus* from different products. A high incidence (53.8%) of enterotoxic *B. cereus* was observed in meat and meat products (Bedi et al. 2004) and from milk and milk products like burfi and skim milk powder (Bedi et al. 2005). Amidst of many reports about toxigenic strains being recorded, Pillai et al. (1993) isolated *B. cereus* cultures from dairy products (lassi, cream and butter) from the city of Madras (now Chennai), India, which were non-enterotoxigenic in nature. In the present study too, not all the 26 isolates characterized biochemically and culturally were toxigenic with respect to haemolysin on blood agar or the presence of toxigenic genes analysed. Radhika et al. (2002) reported the incidence of *B. cereus* strains from traditional Indian food samples exhibiting enterotoxic activity.

4.1.3.3 Molecular basis for relatedness / diversity of selected native food isolates of *B. cereus*

The diversity of the toxigenic strains that exist in the nature is an interesting feature and assumes importance with respect to their documentation and understanding in view of the epidemiological significance. Considering the complex nature of toxigenic factors, for the purpose of determining microbial diversity by genetic analysis, four native food isolates of *B. cereus* (CFR 1529, 1530, 1534 and 1536) were selected based on positive PCR amplification with Ha-1 and Sph primers. The resultant partial

nucleotide sequences of respective PCR amplicons of target genes (*16S rDNA*, *pi-plc*, *hb*l and *sph*) were subjected to BLAST programme of NCBI (online access) to assess the degree of sequence homology. Multiple alignments of the partial nucleotide sequences resulting from PCR amplicons with the corresponding sequences of a few selected strains of *B. cereus* / *B. anthracis* / *B. mycoides* / *B. thuringiensis* are presented in **Tables 5-8**.

Table 5 Multi-alignment of sequences – universal 16S rDNA

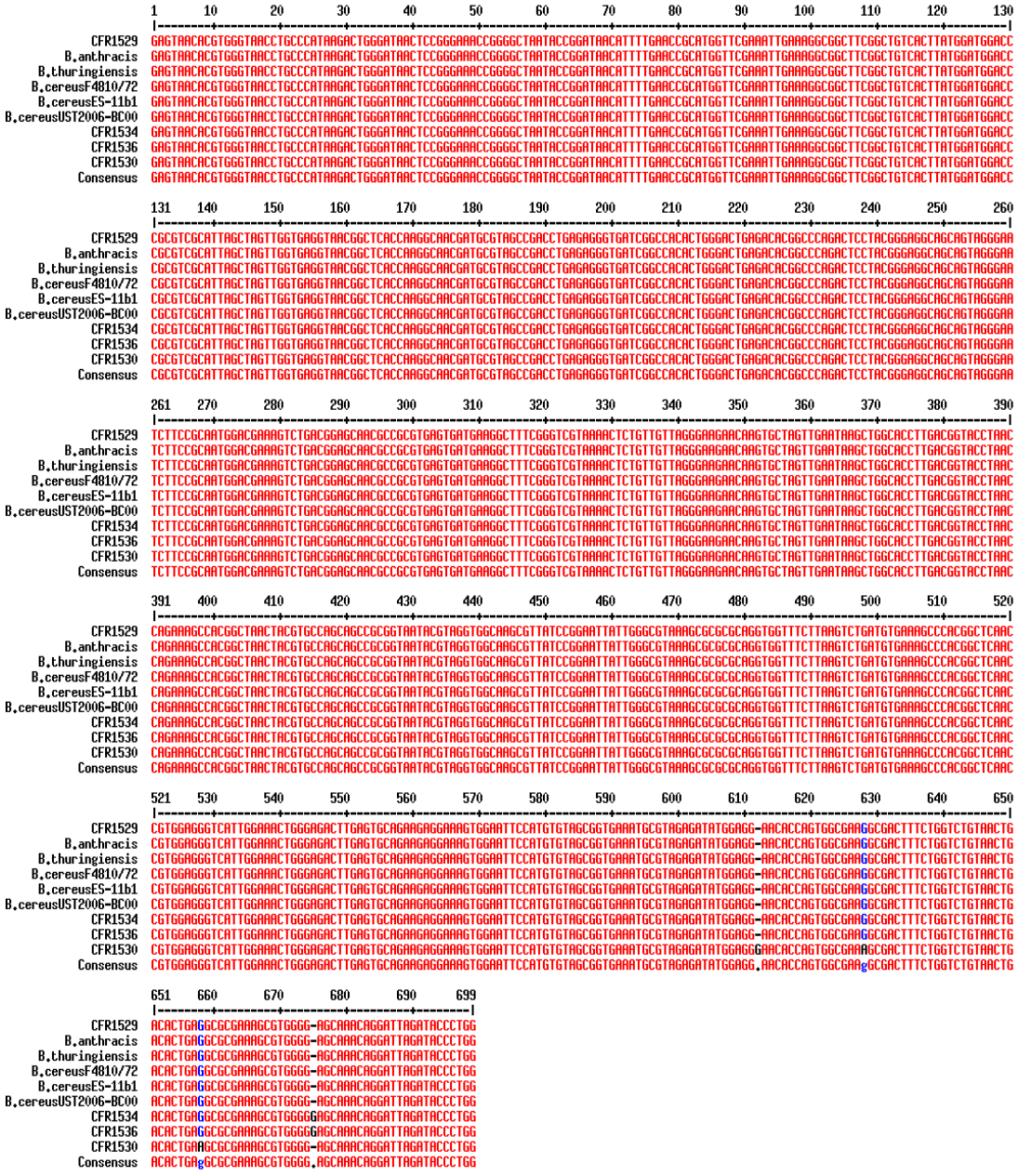


Table 6 Multi-alignment of sequences – Phosphatidylinositol phospholipase C

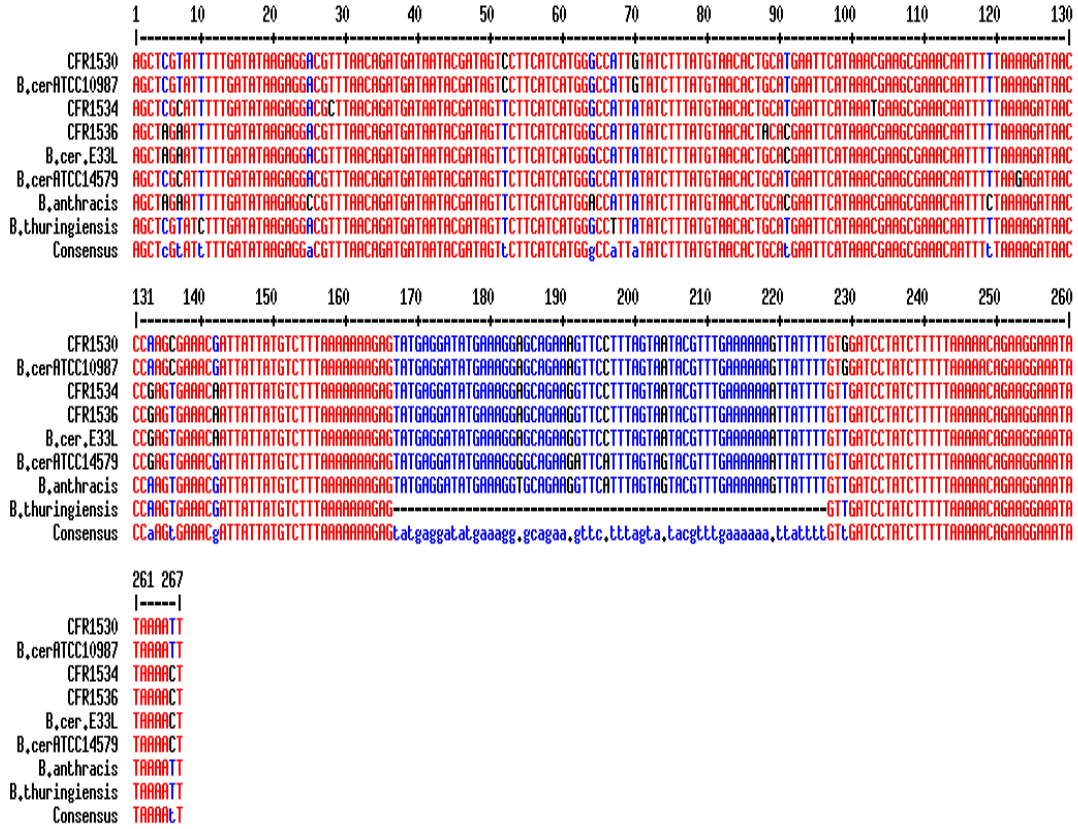


Table 7 Multi-alignment of sequences – Haemolysin BL

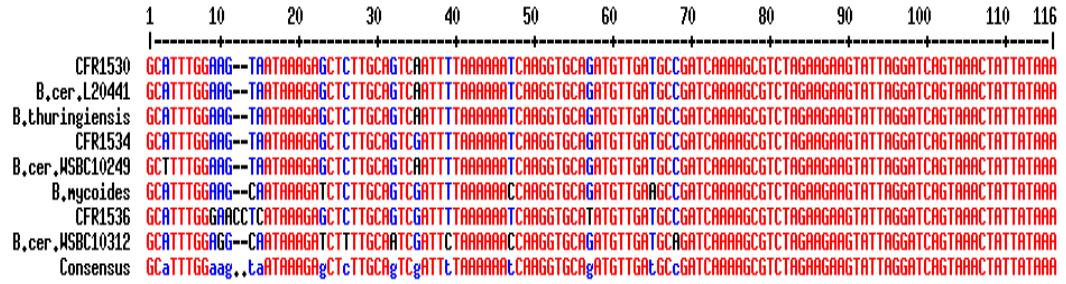
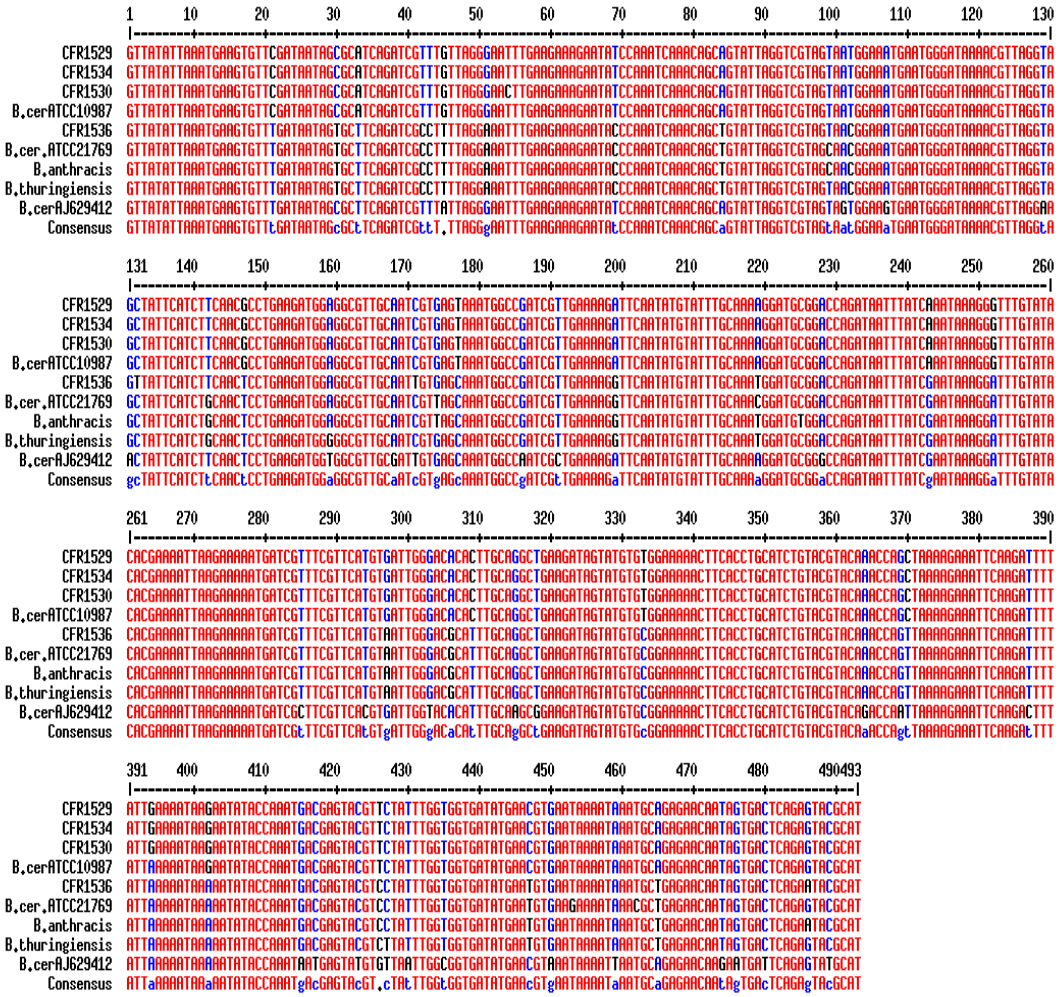


Table 8 Multi-alignment of sequences – Sphingomyelinase



Multiple sequence alignment of the nucleotide sequences resulted in versatile colouring pattern highlighting the conserved sequences (in red coloured font), change in the nucleotide sequence (in blue coloured font) and consensus sequence for the corresponding alignment profile. Very few base pair changes were observed, which indicate a high degree of sequence homology among the aligned nucleotide sequences. This shows that although the strains compared in the alignment were of diverse origin and some belonging to different species altogether, a good degree of agreement existed among the sequences compared within the *B*. *cereus* cluster.

It could be inferred that despite a high degree of homology in the nucleotide sequence of the native isolates with regard to the corresponding genes of the members in the database and a sizeable portion of the sequences showing conserved pattern as seen in multiple alignment, a subtle difference in the sequence would result in a change in the relative position of the native isolate in the phylogenetic tree and thus indicate a corresponding change in the evolutionary relatedness of the organism.

Further, based on these sequences, Force type Neighbour-joining phylograms (**Figures 4-7**) were generated to determine the evolutionary relatedness amongst homologous genes represented in the genome of divergent species. From the generated phylograms, it could be seen that the four native food isolates of *B. cereus* showed a high degree of sequence homology with strains of *B. cereus* as well as other species within the cluster. It was almost 100% in the case of phylogram generated based on the sequence analysis of PCR amplified product of 16S rDNA (**Figure 4**). In an almost similar manner, the homology was in the range of 94-100% in respect of phylogram based on Pi-PLC (**Figure 5**); 92-98% with Ha-1 (**Figure 6**) and 90-99% in the phylogram generated with PCR product from Sph primers (**Figure 7**).

As a means to deduce certain relatedness between the native isolates of *B. cereus* and other cultures appearing in the respective phylograms, details of information pertaining to a few of them documented in literature are summarized in Table 9. In the case of 16S rDNA analysis, a high degree of sequence homology (99%) was observed with 3 strains of *B. cereus*. Similarly, sequence analysis in case of phosphatidylinositol phospholipase C gene generated by species specific primers showed a high homology (99-94%) with strains of *B. cereus* ATCC 14579, E 33L and ATCC 10987. Further, the sequence homology for *B*. *thuringiensis* and *B*. *anthracis* was 97 and 94%, respectively.

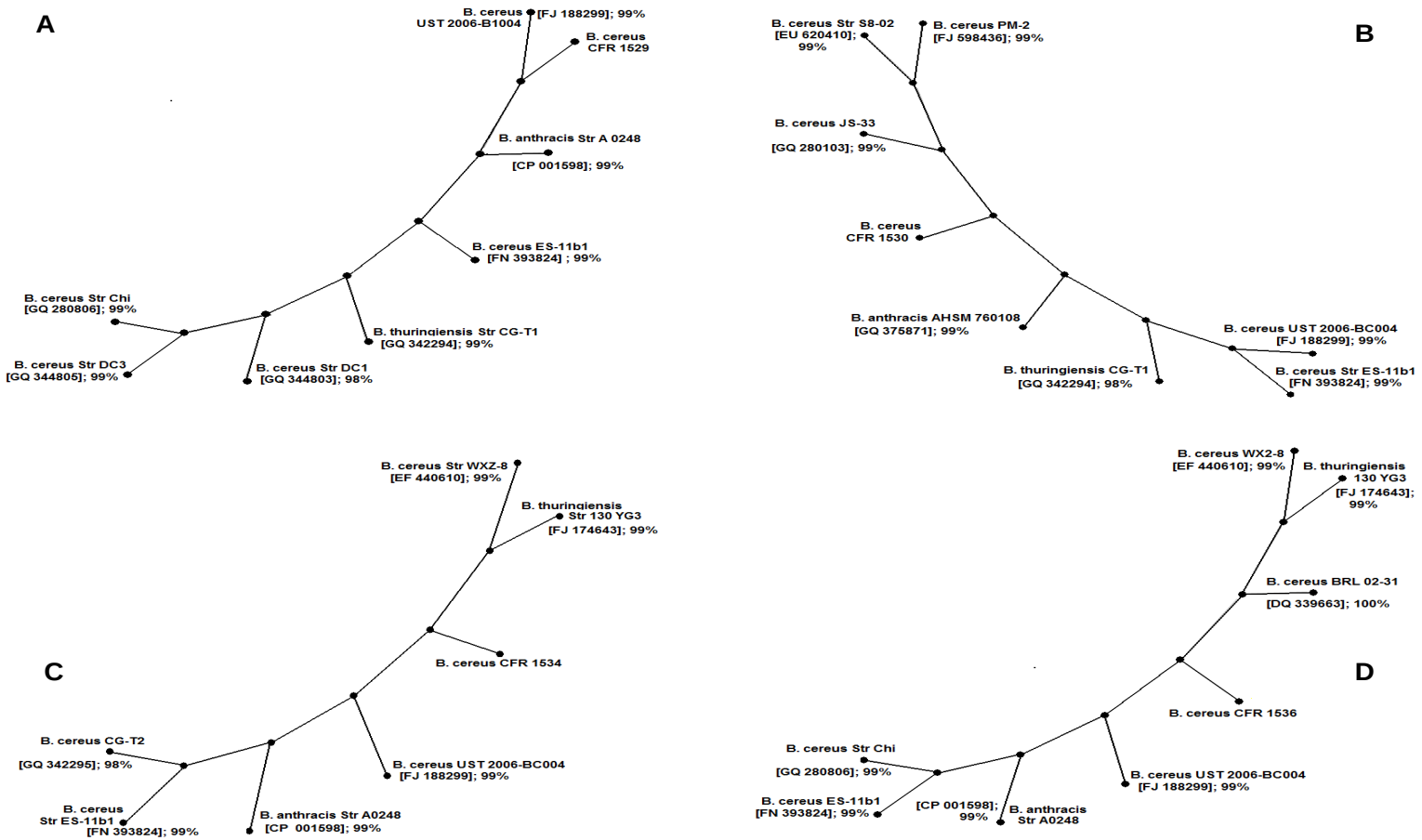


Figure 4 Neighbour-joining phylogram of native isolates of *B. cereus* CFR 1529 [**A**], 1530 [**B**], 1534 [**C**] and 1536 [**D**] based on nucleotide sequence analysis of PCR amplified product with primers of 16S rDNA

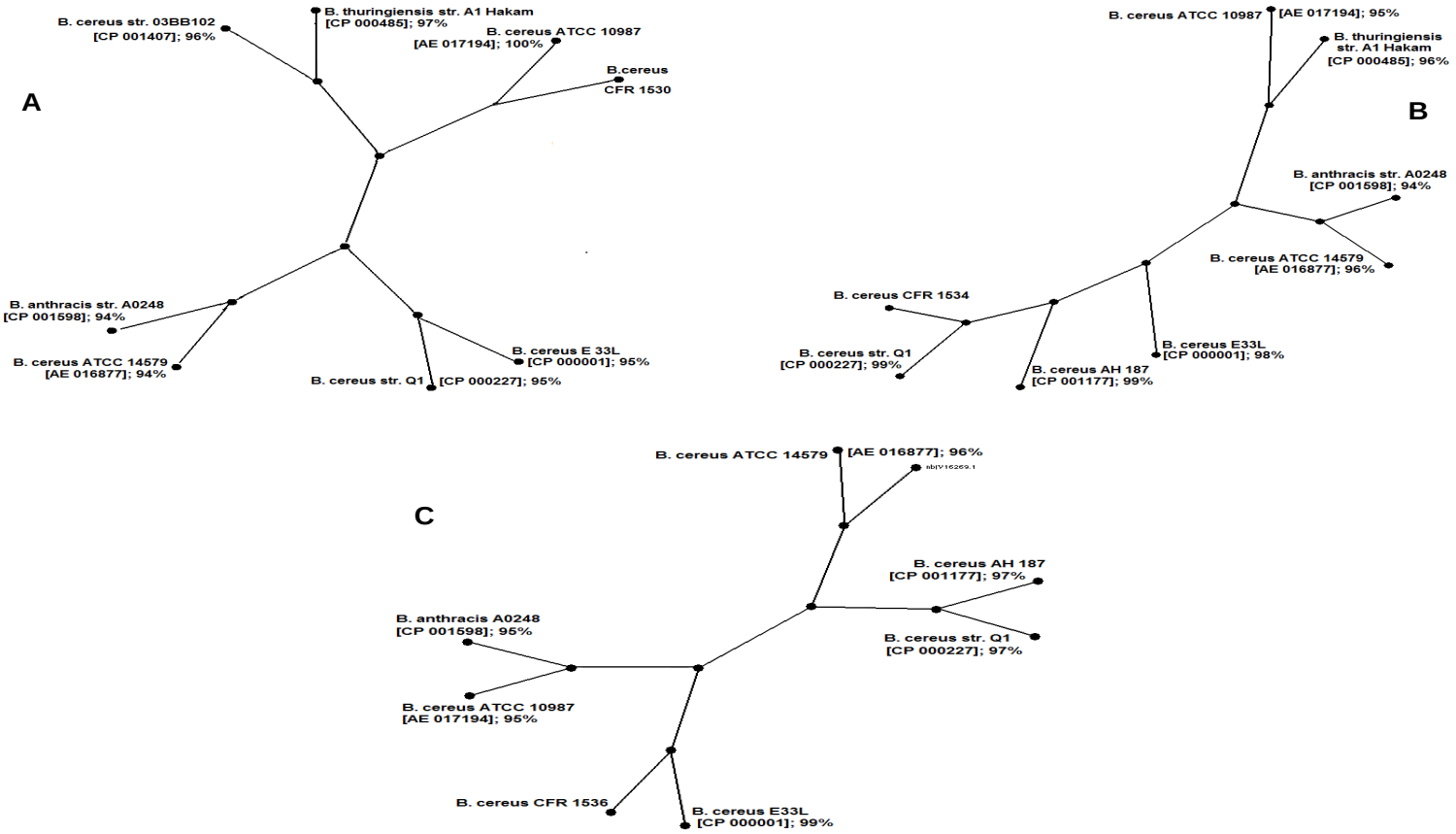


Figure 5 Neighbour-joining phylogram of native isolates of *B. cereus* CFR 1530 [**A**], 1534 [**B**], and 1536 [**C**] based on nucleotide sequence analysis of PCR amplified product with primers of Pi-PLC

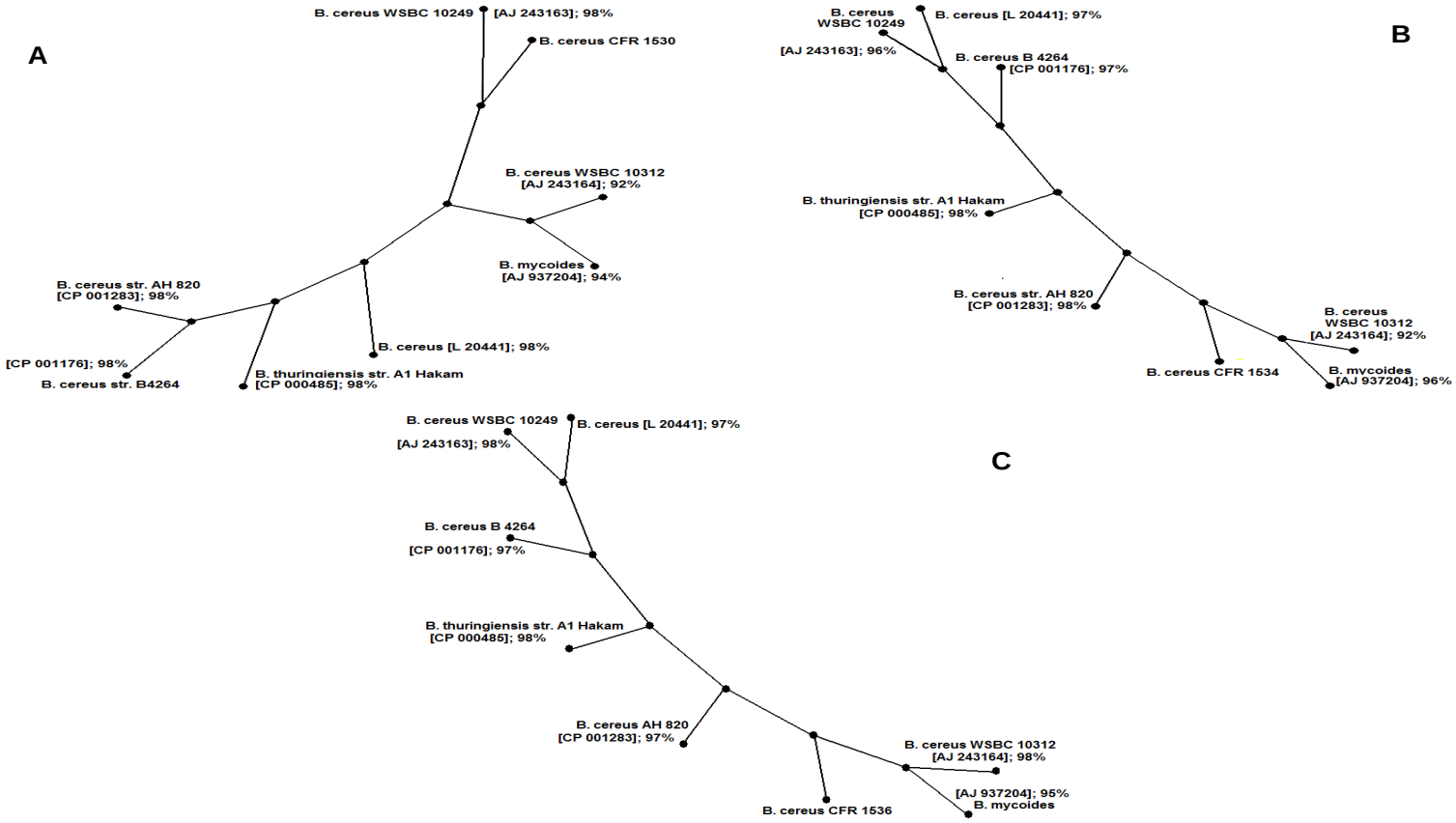


Figure 6 Neighbour-joining phylogram of native isolates of *B. cereus* CFR 1530 [**A**], 1534 [**B**], and 1536 [**C**] based on nucleotide sequence analysis of PCR amplified product with primers of Ha-1

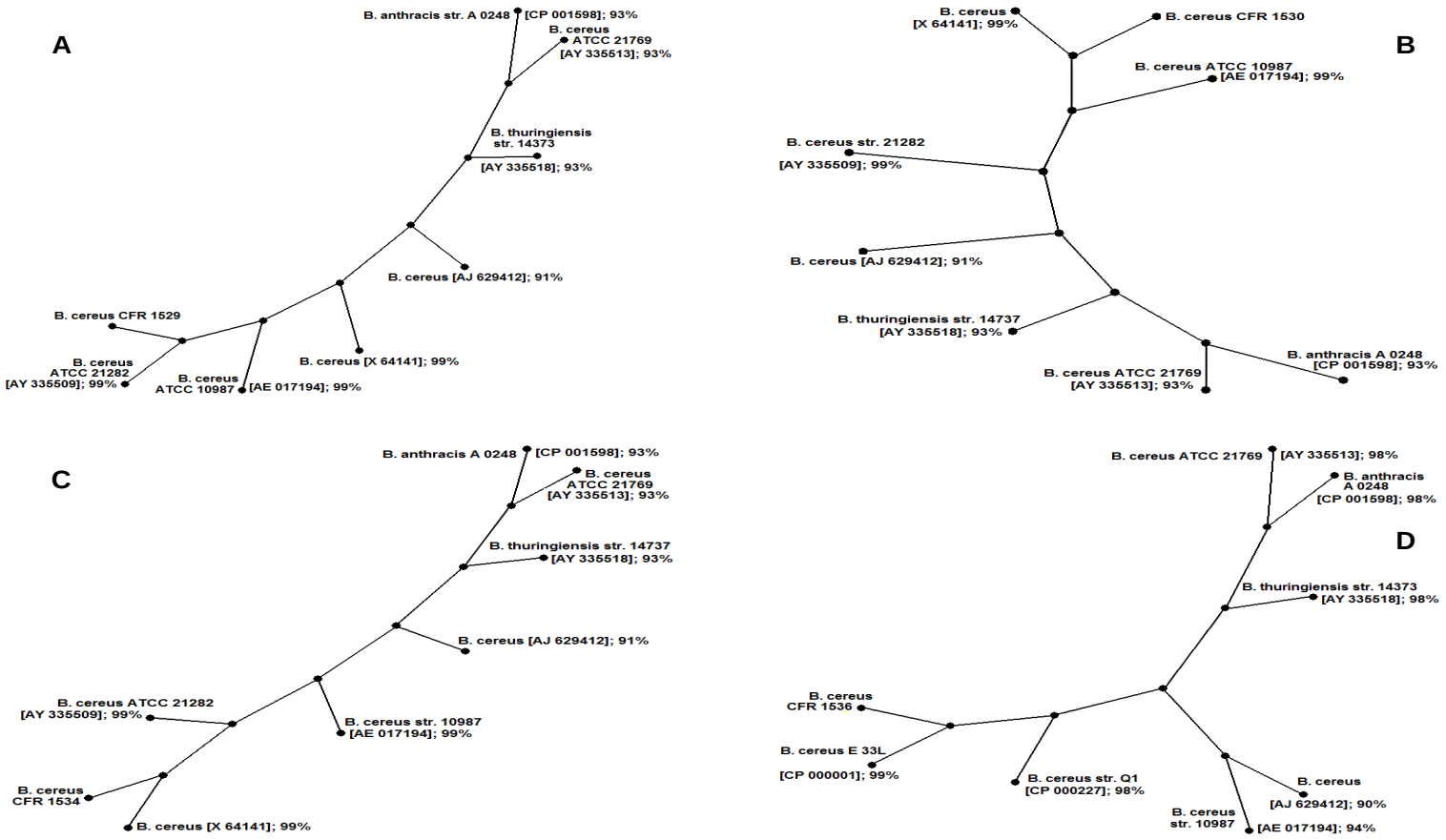


Figure 7 Neighbour-joining phylogram of native isolates of *B. cereus* CFR 1529 [**A**], 1530 [**B**], 1534 [**C**] and 1536 [**D**] based on nucleotide sequence analysis of PCR amplified product with primers of Sph

Table 9 Relatedness among strains of *B. cereus* based on nucleotide sequence homology for specific target genes

Culture / NCBI Acc. No.	Homology / Description	Source of culture	Reference details
B. cereus UST 2006-BC004 FJ188299	99% homology for 16S rDNA with *B. cereus* CFR 1529, 1530, 1534 & 1536	Marine isolate	Ki et al. (2009) J Microbiol Meth 77: 48
B. cereus ES-11b1 FN393824	99% homology for 16S rDNA with *B. cereus* CFR 1529, 1530, 1534 & 1536	Clean room of space craft	www.ncbi.nlm.nih.gov
B. cereus XZM 002 FJ932655	99% homology for 16S rDNA with *B. cereus* CFR 1529, 1530, 1534 & 1536	Aquifier sediments in China	www.ncbi.nlm.nih.gov
B. cereus ATCC 14579 AE016877	99% homology for *pi-plc* with *B. cereus* CFR 1530, 1534 & 1536	Soil dwelling non-emetic	Ivanova et al. (2003) Nature 423: 87 Used as a reference strain
B. cereus E 33L CP000001	95% homology for *pi-plc* with *B. cereus* CFR 1530, 1534 & 1536	Carcass of Zebra	www.ncbi.nlm.nih.gov
B. cereus ATCC 10987 AE017194	94% & 93% homology for *pi-plc* & *sph*, respectively, with *B. cereus* CFR 1529, 1530, 1534 & 1536	Non-lethal dairy isolate in Canada	Rasko et al. (2004) Nucleic Acid Res 32, 977
B. cereus AH 820 / CP001283 *B. cereus* B 4264 / CP001176	98% homology for *hbl* with *B. cereus* CFR 1530, 1534 & 1536	Periodontal pocket and fatal pneumonia patients	www.ncbi.nlm.nih.gov (Unpublished data)
B. cereus F 837/76 L20441	97% homology for *hbl* with *B. cereus* CFR 1530, 1534 & 1536	Surgical wound infection, UK	Heinrichs et al. (1993) J Bacteriol 175, 6760
B. cereus WSBC 10028 AJ 243163	96% homology for *hbl* with *B. cereus* CFR 1530, 1534 & 1536	Soil isolate from Denmark	Damgaard et al. (1996) Syst Appl Microbiol 19, 436
B. cereus ATCC 21769 AY335513	93% homology for *sph* with *B. cereus* CFR 1529, 1530, 1534 & 1536	Chicken and turkey manure	www.ncbi.nlm.nih.gov
B. cereus AJ 629412	87% homology for *sph* with *B. cereus* CFR 1529, 1530, 1534 & 1536	Isolate from larva of Myrmelion bore, Japan	Hisashi et al. (2004) European J Biochem 271, 601

The sequence analysis of haemolysin BL gene revealed a homology of 98-95% with strains of *B. cereus* as well as strains of *B*. *thuringiensis* WS 2734 and *B*. *mycoides* WSBC 10256 (95%) used in multiple alignment analysis. In an almost related manner, there existed a slightly lower (93-87%) homology in the nucleotide sequences of sphingomyelinase gene between the native isolates of *B. cereus* and 3 strains of *B. cereus* as well as *B*. *thuringiensis* and *B*. *anthracis* selected for sequence homology.

Although molecular biology studies of many strains of *B. cereus* cluster are documented and available in public domain, the same is not true with the identity and traceability of culture to its source / origin (habitat and geographical distribution). This aspect is of high importance in microbial diversity and a large number of studies have viewed this lacuna to be of serious concern in conclusively establishing the genetic relatedness and/or diversity among pathogenic cultures. In our study, attempts were made to search for the linkages of sources and habitats of closely related cultures with the four native isolates of *B*. *cereus* used in comparative evaluation of sequence homology. The phylograms generated and multiple alignment sequences for the respective target genes of *16S rDNA*, *pi-plc*, *hbl* and *sph* revealed high degree of homology of the native isolates with strains of *B*. *cereus* and other species of *B*. *cereus* cluster (**Figures 4-7; Table 9**).

In the case of *16S rDNA*, all the four native isolates of *B. cereus* exhibited 99% homology with three strains of *B. cereus*, which had varied isolation sources and geographical location (**Table 9**). In a similar manner, high degree of homology in respect of *pi-plc* was observed for isolates of *B. cereus* CFR 1530, 1534 and 1536 with three strains of *B. cereus* and one strain each of *B*. *thuringiensis* and *B*. *anthracis*. The native isolates obtained in the present study were from rice and milk based foods and appear to be related with non-lethal strains of *B. cereus* associated with soil, dairy and carcass of zebra. The soil dwelling non-emetic strain of *B. cereus* ATCC 14579 has been studied for its complete genome sequence and identifies

genes that are conserved between *B. cereus* and other species in the cluster (Ivanova et al. 2003).

The sequence homology of *hbl* gene revealed a close relatedness of the four native isolates of *B. cereus* with selected strains of *B. cereus* and species of *B. cereus* cluster. In comparison to the native isolates obtained from food related sources, the similarity strains of *B. cereus* for *hbl* gene appear to be predominantly associated with ill-health conditions of human beings, a strong causative factor for the prevalence of this organism in foods through personnel hygienic practices. This highly diversified genetic relatedness does indicate that the haemolysin toxin trait is not only confined to strains of *B. cereus*, but also is prevalent among other species of *B. cereus* cluster (Prüß et al. 1999). In an almost parallel manner, sequence homology observed with closely related strains of *B. cereus* reveal that isolates harbouring *sph* gene tend to be linked with animal habitats. Although one of the closely related strains of *B. cereus* ATCC 10987 is documented as non-lethal dairy isolate, the presence of *sph* gene does not rule out the potential of such strains to be a cause of risk factor to human health. Considering the complexicity of *B. cereus* cluster, a complete comparative genomic analysis has been established, wherein most of the pathogenic traits have been attributed to the presence of plasmids (Rasko et al. 2005; Ehling-Schulz et al. 2006; Rasko et al. 2007).

Studies focusing on evolutionary trends in microbial species based on molecular biology approaches, especially genetic analysis of closely related species are gaining importance in the broad area of microbial diversity (Ochman and Moran 2001). In recent advances of evolution of bacterial genomes, an altered event is usually referred to as ‘indels’ (insertions and deletions) are known to cause frame-shift mutations in bacterial species. This combined with point mutations in the form of base pair substitutions would lead to divergent evolution among closely related species (Britten et al. 2003). In bacterial species, deletions combined with higher number of base pair substitutions are known to occur more frequently than insertions.

The deletional bias also accounts for the compactness of bacterial genomes and not ever-expanding nature, inspite of foreign genes being added up in the form of lateral gene transfer (Mira et al. 2001).

As there have been very few attempts in understanding evolutionary trends in *B. cereus*, in the present study, the pattern of indels observed revealed that were more insertions and base pair substitutions rather than deletions. On the basis of deletional bias as a force towards shaping of bacterial genomes, the observations of our present study may provide an opportunity to suggest that in saprophytic pathogens, there could be a tendency towards stronger selection of pathogenic/virulence traits among the isolates, irrespective of their habitat and geographical distribution. Besides, under these evolutionary changes, there could always be chances for a non-virulent strain to become virulent and vice-versa.

4.1.3.4 Clustering patterns among potential toxigenic native food isolates of *B. cereus* as determined by RAPD-PCR

The ability of native isolates of *B. cereus* to grow at low temperatures showed that 4 isolates (33.3%) grew at 6°C and the other 8 isolates (66.7%) could grow at 10°C. Similarly, evaluation of isolates of *B. cereus* for potent toxigenic traits by PCR as described earlier, revealed that 4 isolates were positive for all the 3 traits tested in this study, while another 4 isolates were positive for Ha-1 and Pi-PLC and one for Sph and Pi-PLC. The remaining 3 isolates were positive only for Pi-PLC. These characteristics exhibited by *B. cereus* isolates have been linked with the source of their isolation, which happens to be food products (**Table 10**). The ability of native food isolates selected in this study did show the ability to grow at low temperatures, wherein foods subjected to cold storage are not devoid from causing health hazards. The potential of these native food isolates in being toxigenic / virulence was evidenced by the presence of either 3 or 2 traits in them by PCR.

Table 10 Characteristics of *B. cereus* isolates with respect to source, growth at low temperatures and toxigenic traits

Isolates of *B. cereus*	Source	Growth at low temp. (°C) 6	10	Toxigenic potential Amplification in PCR Pi-PLC	Ha-1	Sph
CFR 1506	Bread sandwich	+	+	+	+	-
CFR 1508	Bhel puri[a]	+	+	+	+	-
CFR 1521	Pani puri[b]	-	+	+	+	-
CFR 1525	Churi muri[c]	+	+	+	+	-
CFR 1529	Cooked spicy rice	-	-	+	+	+
CFR 1530	Cooked spicy rice	-	-	+	+	+
CFR 1532	Vegetable salad	-	+	+	-	-
CFR 1533	Ice cream	-	-	+	-	-
CFR 1534	Ice cream	+	+	+	+	+
CFR 1535	Khoa[d]	-	-	+	-	+
CFR 1536	Pani puri[b]	-	+	+	+	+
CFR 1540	Raw milk	-	+	+	-	-

[a-c]Processed rice/wheat based foods added with spices and vegetable salads
[d]Heat desiccated milk product used as a base in the preparation of Indian milk sweets

Table 11 RAPD-PCR analysis of native isolates of *B. cereus*

Primer identity	Sequence	No. of bands formed	Amplicon size (bp)
RA-08	TGGCCGTGTG	31	350-1000
RA-14	TTCGAGCCAG	54	210-1000
RA-18	GATGACCGCC	43	200-1000
RA-26	GGACACCACT	40	100-1000

In the background of reproducibility and reliability of RAPD-PCR in differentiating strains of *B. cereus* (Nilsson et al. 1998), preliminary screening of nearly 35 arbitrary primers was performed with two *B. cereus* isolates CFR 1521 and CFR 1534, as a means to assess for their ability to amplify DNA fragments. Based on the visual scoring pattern of bands in two independent sets of experimental trials, 4 primers were selected for RAPD-PCR with all the 12 native isolates of *B. cereus*. The RAPD fingerprints of these isolates with 4 primers can be visualized in gel documentation (**Figure 8**). Each of the 4 primers used generated good number of polymorphic bands and the positions of these bands varied between the isolates. The

total amplified products of the 4 primers was 168 (average of 42 bands per primer), which ranged from almost 100 to 1000 bp (**Table 11**).

The genetic similarity (GS) coefficient for the 12 isolates of *B. cereus* resulting from RAPD analysis ranged from 0.040 (between the isolates CFR 1534 and CFR 1533) to 0.470 (between the isolates CFR 1532 and CFR 1535), except CFR 1540 (**Table 12**). This isolate was different from the other isolates, wherein GS coefficient ranged from 0 (with respect to CFR 1529, CFR 1533 and CFR 1535) to 0.187 (with respect to CFR 1525). Based on the GS coefficient values, the relatedness among 4 potent toxigenic isolates of *B. cereus* namely CFR 1529, 1530, 1534 and 1536 which were found to harbour all the 3 toxigenic traits as assessed earlier in this study was charted out and the GS coefficient was found to be in the range of 0.160 to 0.286.

A broad degree of genetic diversity was observed among the isolates of *B. cereus* subjected to RAPD-PCR analysis, wherein isolates, CFR 1532 and CFR 1535 with highest degree of GS coefficient (0.470) were obtained from two different types of food items namely vegetable salad and a traditional heat desiccated milk product, respectively. Studies by Christiansson et al. (1999) attributed such diverse range of genetic relatedness to the different sampling sites and difference in time of sampling. However, isolates, CFR 1533 and CFR 1534 with least degree of GS coefficient (0.04) were obtained from the same type of food sample, ice-cream, but were placed

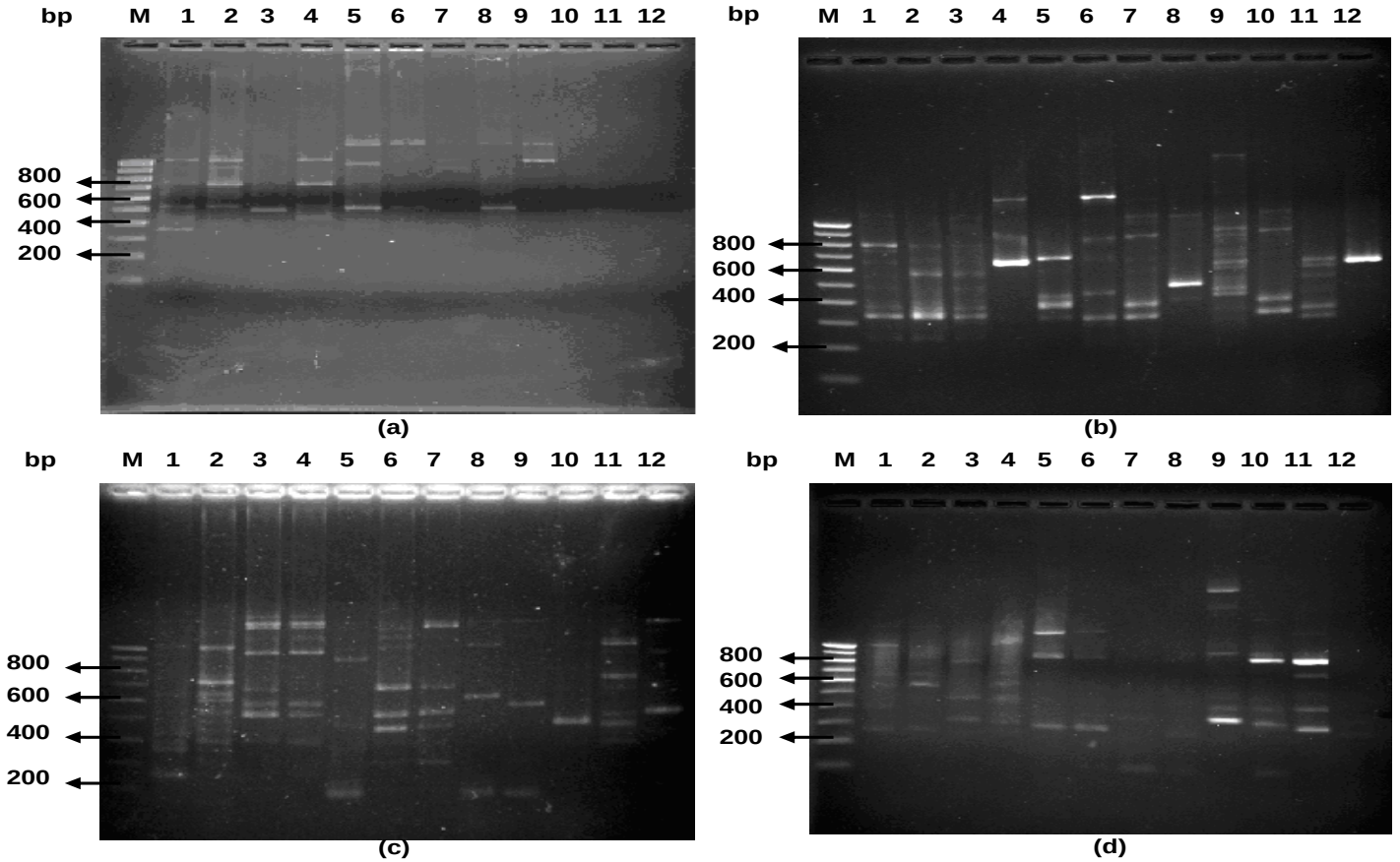


Figure 8 RAPD-PCR profiles of 12 native food isolates of *B. cereus* with randomly selected primers of RA-08 (**a**), RA-14 (**b**), RA-18 (**c**) and RA-26 (**d**).Lane M, DNA ladder of 100-1000 bp; Lanes 1-12, Native isolates of *B. cereus* CFR 1506, 1508, 1521, 1525, 1529, 1530, 1532, 1533, 1534, 1535, 1536 and 1540

Table 12 Genetic similarity coefficient among the native food isolates of *B. cereus* based on RAPD-PCR profiles

Isolates of *B. cereus*	Isolates of *B. cereus* (CFR)											
	1506	1508	1521	1525	1529	1530	1532	1533	1534	1535	1536	1540
CFR 1506	1.000											
CFR 1508	0.417	1.000										
CFR 1521	0.120	0.360	1.000									
CFR 1525	0.120	0.308	0.333	1.000								
CFR 1529	0.227	0.179	0.227	0.080	1.000							
CFR 1530	0.125	0.138	0.227	0.125	0.182	1.000						
CFR 1532	0.182	0.185	0.238	0.130	0.250	0.250	1.000					
CFR 1533	0.043	0.200	0.091	0.043	0.095	0.095	0.100	1.000				
CFR 1534	0.250	0.200	0.250	0.200	0.208	0.160	0.273	0.040	1.000			
CFR 1535	0.125	0.100	0.174	0.080	0.238	0.182	0.470	0.150	0.208	1.000		
CFR 1536	0.214	0.290	0.360	0.259	0.222	0.222	0.185	0.071	0.286	0.222	1.000	
CFR 1540	0.055	0.136	0.055	0.187	0.000	0.059	0.062	0.000	0.105	0.000	0.087	1.000

apart. Similarly, CFR 1529 and CFR 1530 were isolated from processed rice based product and had a low degree of GS coefficient (0.182). The RAPD-PCR pattern provides evidence toward the existence of relatedness and diversity. In a few of the earlier studies, RAPD-PCR enabled detection of contamination of *B. cereus* in food ingredients, finished product and processing plant based on genetic diversity (Christiansson et al. 1999). The present study revealed that irrespective of isolation source of *B. cereus* cultures (**Table 10**), there did exist genetic relatedness among 4 native isolates of *B. cereus*, which harboured all the 3 potent toxigenic traits as determined by PCR.

The dendrogram generated based on GS coefficients of RAPD analysis showed clear distinction into major and minor clusters (**Figure 9**) with the presence of 3 major clusters A, B and C and sub-clusters within the major clusters. The sub-clusters, A1 and A2 had 2 and 4 isolates, respectively, while B1 had 3 isolates and B2 one isolate. Cluster C had only one isolate. The native isolate of *B. cereus* CFR 1540 was not present among the clusters generated and remained as a non-cluster pattern. In the present study, as a means to understand the possibility of any linkage factors, an attempt is being made to bring out certain relationships among the native food isolates of *B. cereus*.

Another attribute being addressed, is the processing criteria involved during the preparation of food that has been the source of isolates. Nearly 66.6% of isolates in cluster A were obtained from a similar type of food product based on processed rice/wheat added with spices and vegetable salads and devoid of any heat processing, but was a mere mixing of ingredients at the point of consumption. An almost identical situation did exist in case of 2 other isolates of cluster A. In cluster B, 75% of the isolates were obtained from foods, which had undergone at least one step of heat processing and the remaining isolate was from a product devoid of any heat processing. Similarly, isolate in cluster C and the one under non-cluster have been from food products not exposed to any heat processing. In an identical scenario,

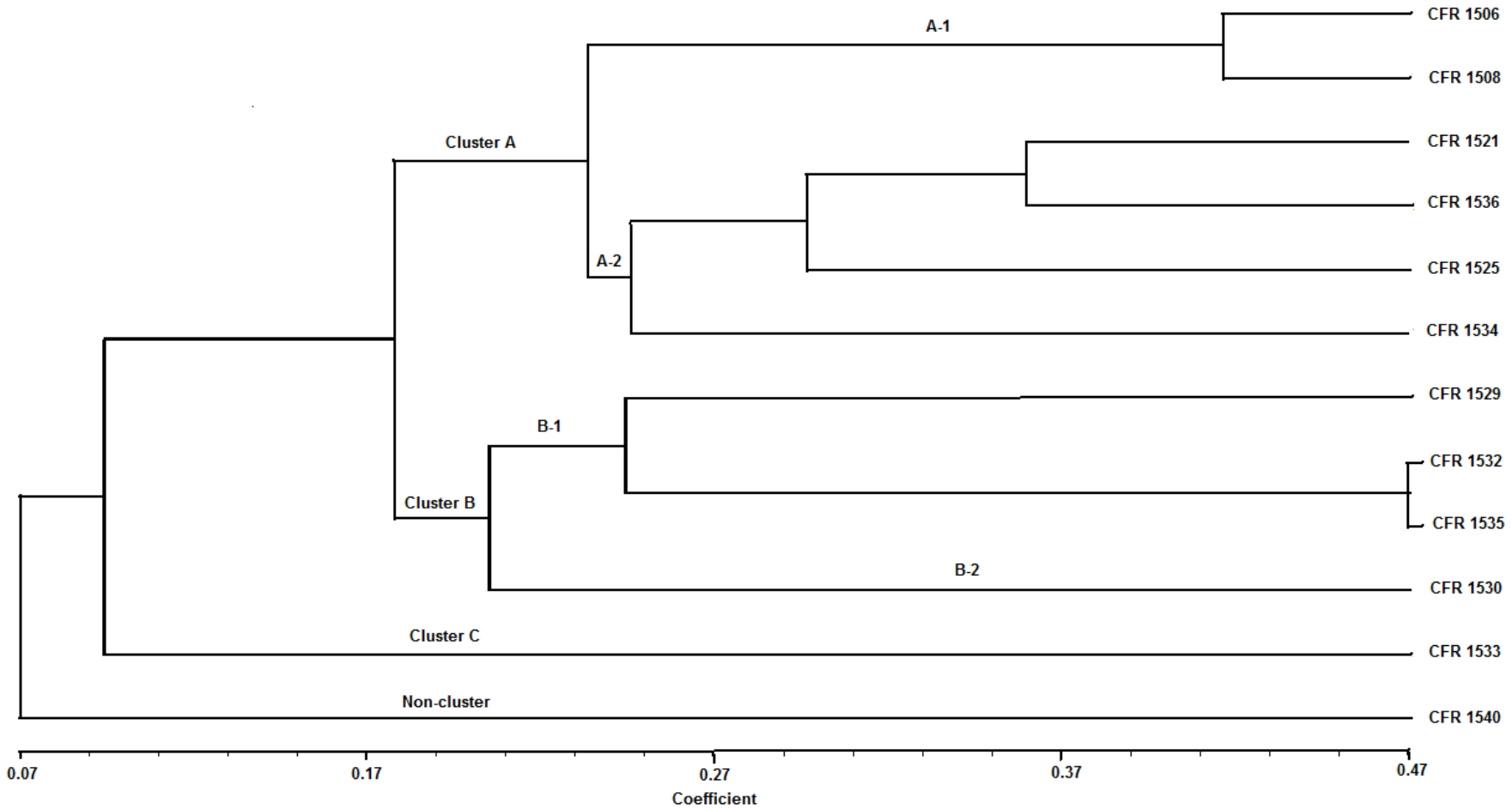


Figure 9 Dendogram showing genetic relatedness among the native isolates of *B. cereus* based on the cluster analysis of RAPD-PCR data

RAPD has served as a useful genotyping technique to identify specific subtypes associated with specific source and location with respect to epidemiological investigations relating to strains of *Salmonella enteritidis* and *S*. Typhimurium occurring in Italy (De Cesare et al. 2001).

Another aspect of approach was the growth of *B. cereus* isolates at low temperature. All the isolates of *B. cereus* in cluster A were able to grow at 6 and/or 10°C, indicating almost psychrotrophic nature of these cultures (**Table 10**). Except for *B. cereus* CFR 1532 (growth at 10°C) in cluster B, other remaining isolates in cluster B and the one isolate of cluster C were mesophilic. The isolate of *B. cereus* CFR 1540 present under non-cluster had a growth temperature of 10°C. As a means to interlink the basis of source of isolation and growth temperature, it becomes evident that isolates of *B. cereus* obtained from foods which had a unit operation of heat processing (cluster B) were mesophilic. Similarly, *B. cereus* cultures obtained from foods which did not involve heat processing at the time of consumption (cluster A) were psychrotrophs. In a similar approach to the present study, RAPD analysis using 3 different primers could clearly distinguish psychrotolerant strains of *B. cereus* group which resulted in proposing a new species *B*. *weihenstephanensis* sp. nov. (Lechner et al. 1998). On the same lines, studies by Svensson et al. (1999) resulted in an identical banding profile of psychrotrophic strains of *B. cereus* from production line of a dairy. Similarly, Gaviria and Priest (2003) observed that although a high level of genomic homogeneity existed among the different *B*. *thuringiensis* strains, most of the serovars included at least one variant strain.

The results of the present study correlates well with the cluster analysis pattern performed by Hammer et al. (2001) wherein RAPD profile of *B. cereus* strains from a milk powder plant revealed no perfect similarity between the strains analyzed. Like-wise Ghelardi et al. (2002) confirmed the genomic heterogeneity for toxigenic strains of *B. cereus* implicated in two food poisoning outbreaks wherein the amplification pattern generated were distinct for all of the *B. cereus* strains analyzed.

RAPD although is a rapid, inexpensive and technically feasible tool, it has a disadvantage of poor reproducibility, especially when performed in different laboratories. In this context, studies by Levy et al. (2005) assumes significance in that the strains of *B. anthracis* were not only successfully characterized but also cloned and sequenced specific RAPD markers that enabled the classification of strains into distinct clusters with better reproducibility.

A more probable basis of relatedness has been the prevalence of toxigenic traits among the native isolates of *B. cereus*, particularly those of Ha-1 and/or Sph, as all isolates were positive for Pi-PLC (**Table 10**). It is of interest to record that all isolates of *B. cereus* in clusters A and B did harbour the toxigenic trait of Ha-1 and/or Sph. However, isolates in cluster C and non-cluster were negative for both the toxigenic traits. The findings did bring out that the presence of one or more of these toxigenic attributes in the isolates is a difficult proposition to analyze and there appears to be no single directional approach. Earlier research investigations on similar lines have clearly revealed that RAPD-PCR analysis could differentiate groups of isolates of *B. cereus*, *B*. *anthracis*, *B*. *thuringiensis* and other species based on combination of phenotypic and genotypic traits (Ghelardi et al. 2002; Gaviria et al. 2003; Levy et al. 2005).

In an approach towards projecting the importance of clustering pattern, a hypothetical scheme was prepared (**Figure 10**) based on prevalence of toxigenic traits in the isolates of *B. cereus* and the source of their isolation (food products). The scheme projects the significance of clustering pattern, wherein it is evident that isolates of *B. cereus* obtained from specific types of traditional fast foods did harbour potent toxigenic traits and occurrence under one cluster like cluster A (**Figure 9**). However, milk based foods did not reveal any specific linkage with cluster pattern as isolates of *B. cereus* obtained from such foods did include clusters A, B, C and non-cluster. To a certain extent, spicy and vegetable based foods did have relatedness with *B. cereus* isolates of cluster B pattern. The scheme presented here may have

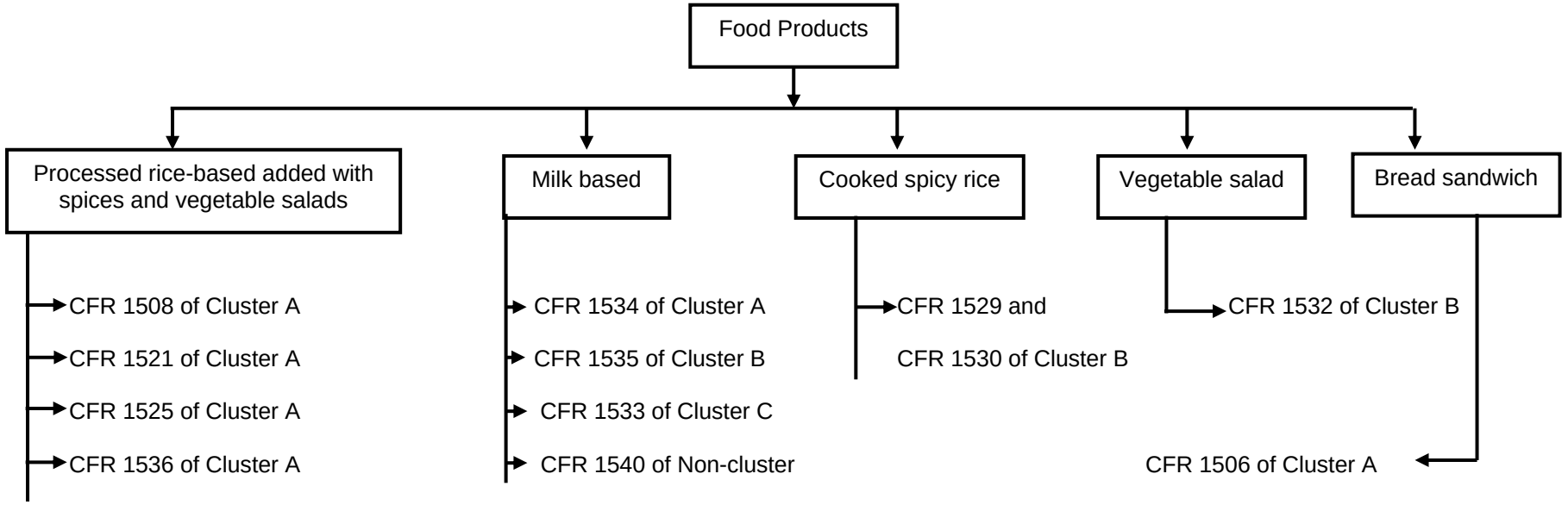


Figure 10 Hypothetical representation of clustering pattern among potent native toxigenic isolates of *B*. *cereus* in relationship to their source

limitations for wider applications as large number of isolates and specific type of foods associated with their isolation are to become the subject of study.

The findings of present study based on molecular biology approach indicated the genetic relatedness among the native isolates and toxigenic traits harboured in them, irrespective of the geographical boundaries and isolation sources.

4.2 THERMAL INACTIVATION PROFILE OF VEGETATIVE CELLS AND SPORES OF *BACILLUS CEREUS* SUBJECTED TO SIMULATED TIME-TEMPERATURE COMBINATION OF INDIAN TRADITIONAL FOODS

In the present global scenario, consumer preferences are on an increase towards minimally heat processed foods. Besides, several newer brands of food products (Ready-to-Eat and Ready-to-Cook) have arrived on the market shelves, which are mostly semi-processed and needs additional one or two steps of heat processing of varying degrees prior to consumption by human population. At the same time, concern for microbial safety of foods and the risk involved due to the opportunistic foodborne pathogen, *Bacillus cereus* attains greater significance in view of the dual phase of this pathogen i.e. vegetative and spore phases. Although pasteurization is known to inactivate the vegetative cells, a few aberrations in the time-temperature combinations during thermal processing of foods, combined with ample opportunities of post-processing contamination underscores the need to assess the thermal resistance of vegetative cells.

In contrast, spores of *B. cereus* are known to be highly heat resistant and are usually unaffected by physical, chemical and biological factors encountered in a food chain. The spores that survive pasteurization can germinate and outgrow to produce enterotoxins in foods, which may lead to foodborne illness. Studies relating to the effect of heat processing schedules on spores of *B*. *cereus* have attempted to focus on specificity of strains as well as cultural, physiological and biochemical attributes. However, it would be of significance, if both the phases of pathogenic organisms are assessed in relevance to the scenario of microbial safety in food chain, as the behaviour of an organism is mainly dependent on culture identity.

In this background, the objective of present study was to assess the thermal inactivation profile in terms of *D*- and z-values of vegetative cells and spores of 3 potent

toxigenic native food isolates of *B*. *cereus* subjected to heat treatments known to occur in traditional food chain operations. *D*-value is the decimal reduction in time (in min) that is needed to reduce the viable cell population by one log. The *z*-value is the temperature needed to reduce the D-value by 10-fold.

4.2.1 MATERIALS

All glasswares, media and other materials used in the present study were either wet sterilized or dry sterilized. Wet sterilization was carried out at 121°C for 20 min in an autoclave and dry sterilization at 180°C for 4 h in a Hot Air Oven. All bacteriological media used were those of dehydrated media procured from Hi-Media Lab., Mumbai, India (Hi-Media, 2003). The media were prepared as per manufacturer's instructions. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

4.2.1.1 Bacterial (test) cultures

These included 3 potent toxigenic native food isolates of *B*. *cereus* (CFR 1521, CFR 1532 and CFR 1534) selected from the Departmental collection of bacterial cultures that have been isolated from a range of Indian traditional fast foods collected from the local markets of Mysore City, India. In addition, strain of *B*. *cereus* F 4810 obtained through the courtesy of Dr. J.H. Kramer, Central Public Health Laboratory, London, United Kingdom served as the reference culture. The cultures were individually maintained at 6°C on Brain Heart Infusion (BHI) agar slants and the cultures were propagated in BHI broth for 18 h at 37°C, prior to use in experimental trials.

4.2.1.2 Normal saline

The normal saline of 0.85% (also used as diluent) was prepared as described under **4.1.1.3.1**. Prepared saline was dispensed in requisite quantities in suitable glass containers and autoclaved.

4.2.1.3 Skim milk

This was prepared by reconstituting requisite quantities of spray dried skim milk powder (quality brand of a commercial dairy in India) with water to a final level of 10% (w/v). The reconstituted milk was filtered through moistened cotton pad, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity as well as in 10 ml amounts in test tubes (18 x 150 mm) and autoclaved at 121°C for 15 min.

4.2.1.4 Whole milk

Pasteurized milk of 3.0% fat content was procured from the local commercial Dairy (Mysore Dairy, Mysore, India) retail outlets, filtered through moistened cotton pad, dispensed in requisite quantities in Erlenmeyer conical flasks of suitable capacity as well as in 10 ml amounts in test tubes (18 x 150 mm) and autoclaved at 121°C for 15 min.

4.2.1.5 Brain heart infusion (BHI) broth and agar

This was prepared as described under **4.1.1.7.3** and used in this study.

4.2.1.6 Plate count agar

The medium composition is as follows (g/l):

Casein enzymic hydrolysate	5.0
Yeast extract	2.5
Dextrose	1.0
Agar	12.0
Final pH 7.2 ± 0.2	

The requisite quantity of dehydrated medium was dissolved in water by boiling, dispensed in appropriate amounts in Erlenmeyer conical flasks and autoclaved.

4.2.2 METHODOLOGY

4.4.2.1 Heating menstra

The heating menstra used in this study were normal saline, BHI broth, skim milk and whole milk. Normal saline served as a control medium without any complex nutrient ingredients. BHI broth was used as a representative of routinely used laboratory complex microbiological general purpose medium. Reconstituted skim milk and whole milk of 3% fat served as media with food constituents.

4.2.2.2 Preparation of vegetative cells

A loopful of test cultures from individual active agar slants were inoculated into aliquots of 10 ml of BHI broth and incubated for 14 h at 37°C in an orbital shaker incubator (Alpha Scientific Co., Bangalore, India) at 70 rpm. A smear of loopful of individual culture broths was observed under a laboratory compound microscope to rule out the presence of any spores. Cell suspensions of the cultures were individually prepared by centrifugation at 8000 rpm (Superspin R-V/F_M, Plasto Crafts, Mumbai, India) for 20 min at 4°C, followed by washing of harvested cells twice with 0.85% saline and resuspending in 10 ml saline. The cell titre was enumerated by surface plating of serial dilutions of the initial cell suspension on pre-poured BHI agar plates and incubated for 24 h at 37°C. The concentration of cells was 9.3 $\log_{10}$ CFU/ml, stored at 6°C in aliquots of 10 ml of 0.85% saline in sterile screw-capped tubes until further use.

4.2.2.3 Preparation of spore suspension

Spore suspensions of individual isolates of *B*. *cereus* were prepared in accordance with the procedure of Novak et al. (2005). A loopful of culture from individual active agar slants was inoculated into 10 ml of BHI broth and incubated for 18 h at 37°C under static condition. The culture broths were diluted in 0.85% saline and surface plated on pre-poured BHI agar plates containing 3 mg/ml $MgSO_4$. The plates were incubated for 72 h at 37°C and subsequently smears of the colonies were observed under a laboratory compound microscope for more than 90% sporulation. Spores were harvested from multiple plates by gentle scrapping with sterile L-shaped spreaders, pooling, washing and resuspending in 10 ml aliquots of 0.85% saline and ethanol was at a concentration of 20% v/v. The spore titre was determined as described previously for cell titre of vegetative cells and final concentration adjusted to 9.3 $\log_{10}$ spores/ml. The spore suspensions were stored at 6°C in sterile screw-capped tubes until further use.

4.2.2.4. Thermal inactivation and enumeration of survivors

Individual pre-sterilized heating menstra were taken in aliquots of 5 ml in test tubes of 15 x 125 mm and placed initially at pre-selected temperatures in a thermostatically controlled water bath (Julabo SW 22, Labortechnik GMBH, Seelbach, Germany), so that the menstra attains the defined temperature to be used in this experimental trials. These tempered aliquots of individual heating menstra were inoculated with vegetative cells of individual bacterial test cultures of *B*. *cereus* at a level of 8.3 $\log_{10}$ CFU/ml. The inoculated tubes were subjected to temperatures of 56, 58 and 60°C, respectively, for specific durations of 3, 6, 9, 12, 15 and 18 min. After the specific time-temperature periods were completed, tubes were removed and immediately cooled in ice-water bath. The experimental samples were appropriately diluted in 0.85% saline and surface plated on pre-poured plates of BHI agar and incubated aerobically for 24 h at 37 °C. Colonies

formed in the incubated plates were enumerated and recorded in $\log_{10}$ CFU/ml. Similar heat treatment and enumeration procedures as described for vegetative cells were performed in the four individual heating menstra for spores of individual test cultures of *B*. *cereus* at pre-selected temperatures of 85, 90 and 95°C, respectively, for specific durations of 5, 10, 15, 20, 25 and 30 min. The results recorded were the average of two experimental trials and statistically analyzed as per the requirement of experimental plan and presentation.

4.2.2.5. Determination of thermal resistance and statistical analysis

The *D*-values (time in min) required to reduce the viable cell population by 90% were determined by separately plotting the $\log_{10}$ number of survivors against time at each temperature using Microsoft excel 2003 software (Microsoft Corporation, Redmond, USA). Only the values in straight portion of the curve were considered for calculation. The line-of-best fit for survivor plots were determined by regression analysis (Ostle and Malone, 1988). A regression equation of the type y =a + bx was derived, where b is the slope of the best straight line that when inverted and changed from negative to positive, gives the *D*–value for a specific temperature (D = -1/slope). The *D*-values were calculated using the average slope of the 2 sets of experiment performed for each temperature. The *z*-values were obtained by plotting the $\log_{10}$ *D*–values against respective temperatures and calculating the negative inverse of the slope of the curve (Bolton et al. 2003).

The significant difference in *D*-values among the different strains tested was performed by Duncan's New Multiple Range Test. One-way-analysis of variance of mean *D*-values of the cultures across heating media and temperatures as well as that with respect to cultures and temperatures was performed using Origin Pro 6.1 software (Origin Lab Corporation, Northampton MA, USA).

4.2.3 RESULTS AND DISCUSSION

4.2.3.1 Thermal inactivation profile of vegetative cells

Decimal reduction times of vegetative cells of the individual cultures of *B*. *cereus* in selected heating menstra and their corresponding 12 *D*-values are presented in **Table 13**. The inactivation pattern for vegetative cells was sigmoidal with R^2 (correlation coefficient) of 0.95. As the pattern was almost same in all the 4 cultures studied for all the different heating menstra at varied temperatures, the sigmoidal curve for only one representative culture of *B*. *cereus* CFR 1534 (a potent toxigenic isolate) is shown in **Figure 11**. The *D*-values for the 4 strains of vegetative cells across the different menstra ranged from the lowest of 3.45 min at 60ºC to the highest of 10.6 min at 56ºC in saline. The findings of present study with native food isolates of *B*. *cereus* known to occur in a wide variety of traditional foods gains significance in the background of not many reports available with respect to the thermal inactivation of vegetative cells of *B. cereus*. In a similar experimental approach with almost different type of heating menstruum (pork luncheon roll), the values recorded were from a lowest of 1 min at 60ºC to a highest of 6.4 min at 55ºC (Byrne et al. 2006). The findings of present study with native food isolates of *B*. *cereus* known to occur in a wide variety of traditional foods gains significance in the background of not many reports available with respect to the thermal inactivation of vegetative cells of *B. cereus* although literature is well documented with information on thermal inactivation of spores.

The *D*-values observed in the present study appear to be quite different from those reported by earlier researchers. This variation could be attributed to the genotypic characteristics of the bacterial cultures and basic experimental design used in the respective studies. The predominant influencing factor appears to arise from the biochemical and physical constituents like levels of carbohydrates, fat and protein in heating menstra, which could enhance thermal

Table 13 Decimal reduction time* (*D*-values) of vegetative cells of *B. cereus* cultures

Culture	Heating menstra											
	Saline			BHI broth			Skim milk			Whole milk		
	56°C	58°C	60°C	56°C	58°C	60°C	56°C	58°C	60°C	56°C	58°C	60°C
	(Time, min)											
B. cereus CFR 1521	7.40[a]	3.65[a]	3.50[a]	10.55[a]	3.50[a]	4.05[a]	7.60[a]	6.90[a]	3.70[a]	7.65[a]	5.45[a]	3.60[a]
	(88.8)	(44.4)	(42.0)+	(127.2)	(42.0)	(49.2)	(91.2)	(82.8)	(44.4)	(92.4)	(66.0)	(43.2)
B. cereus CFR 1532	7.20[a]	4.85[b]	3.45[a]	6.20[b]	4.25[ab]	3.50[a]	5.30[b]	4.75[b]	3.45[a]	5.15[b]	4.10[a]	3.55[a]
	(86.4)	(58.8)	(42.0)	(74.4)	(51.6)	(42.0)	(63.6)	(57.6)	(42.0)	(62.4)	(49.2)	(43.2)
B. cereus CFR 1534	10.60[b]	4.90[b]	4.10[a]	8.05[ab]	5.80[c]	4.15[a]	7.15[a]	5.45[b]	3.90[a]	6.50[c]	4.75[a]	4.15[a]
	(127.2)	(58.8)	(49.2)	(97.2)	(69.6)	(50.4)	(86.4)	(66.0)	(46.8)	(78.0)	(57.6)	(50.4)
B. cereus F 4810	7.70[a]	5.45[c]	3.90[a]	7.00[b]	5.25[bc]	3.65[a]	7.65[a]	4.65[b]	4.25[a]	6.50[c]	4.90[a]	4.15[a]
	(97.2)	(66.0)	(46.8)	(84.0)	(63.6)	(44.4)	(92.4)	(56.4)	(51.6)	(78.0)	(58.8)	(50.4)
SEM	±0.43	±0.12	±0.12	±0.67	±0.30	±0.39	±0.32	±0.26	±0.21	±0.16	±0.45	±0.22

[a-c]Means of the same column followed by different letters differ significantly (P <0.05) according to Duncan's New Multiple Range Test

**D*-values are the means of two experimental trials, with each performed in duplicates

+Figures in parentheses indicate the 12D (commercial sterility) values

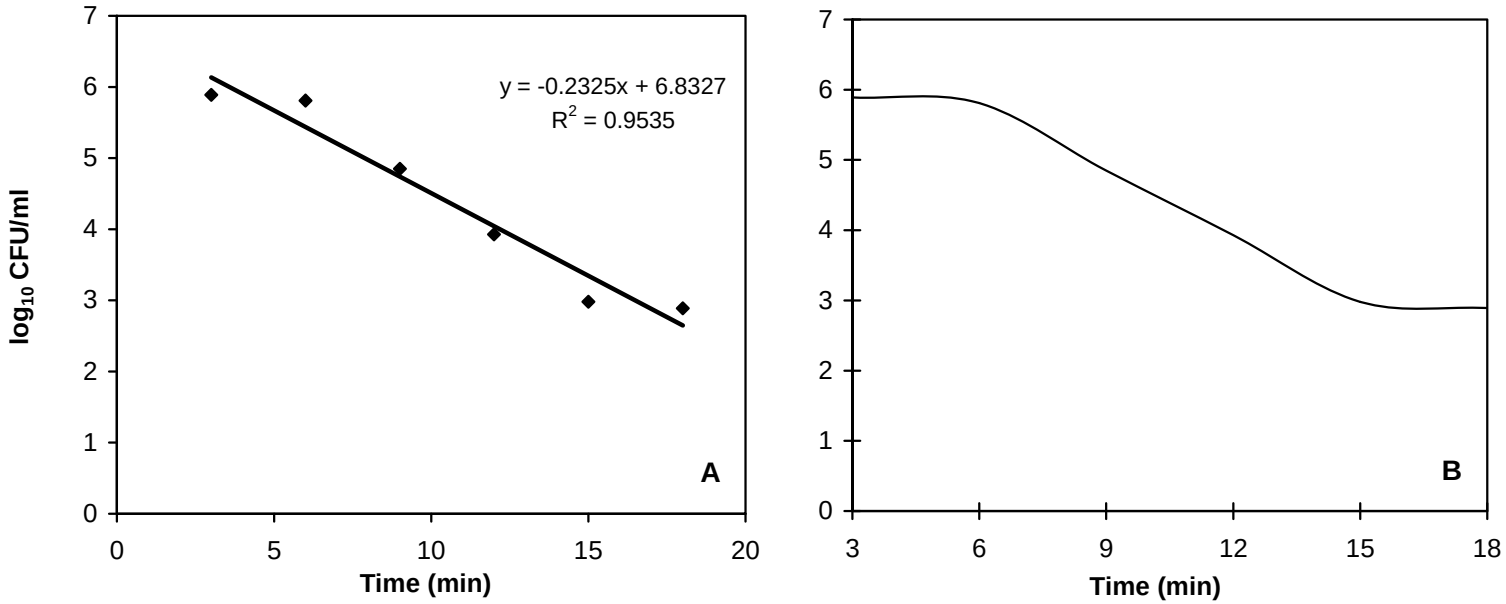


Figure 11 Survivor curves for vegetative cells of *B*. *cereus* CFR 1534 in skim milk at 60°C showing the line of best fit [**A**] and sigmoidal curve [**B**]

resistance (Ababouch et al. 1995; Otieza et al. 2003) and so also higher acidic environment (low pH levels) has a similar effect (Casadei et al. 2001). In contrast to this, an increase in water activity was found to lower *D*-values (Gaillard et al. 1998). Usually, the differences being observed have been due to the effect of combination of these influencing factors.

Considering the behavioural pattern of isolates of *B*. *cereus* used in the present study, the findings revealed that there was no marked difference in the thermal resistance of vegetative cells of these cultures ($p < 0.05$). The isolate of *B*. *cereus* CFR 1532 with a mean *D*-value of 4.7 min was resistant as compared to the other cultures studied (**Figure 12**). Similarly, all the 4 heating media had no marked differential effect on thermal resistance pattern of vegetative cells (**Figure 13**). The mean *D*-values for saline, BHI broth, Skim milk and whole milk were 5.6, 5.5, 5.4 and 5.1 min, respectively and were statistically not significant ($p < 0.05$). This shows that though whole milk had a fat content of 3%, it did not play any role in imparting protection against heat. This is against the general notion derived from earlier studies, that fat, often protects the organism against heat. A study on thermal resistance of *Listeria monocytogenes* in dairy products had shown a similar phenomenon, wherein fat had no effect on heat resistance (Casadei et al. 1998).

Juneja and Marmer (1998) reported *D*-values in *Clostridium perfringens* as 3.5 min at 60 °C and 14.5 min. at 55 °C in beef supplemented with 0.15% Sodium pyrophosphate (SPP) and turkey with 0.3% SPP, respectively, the values which are concordant with those recorded in the present work. Although no direct comparison could be done for the inactivation values between meat products and heating menstra studied, in the absence of many reports, an analogy was drawn owing to the Gram positive nature and endospore forming ability of the *C. perfringens.* Similarly, *D*-values of

E. coli O157:H7 at 58°C in nutrient broth (which is close to BHI in terms of consistency and composition) was 1.6 min, which is less compared to 5.5 min in the present study.

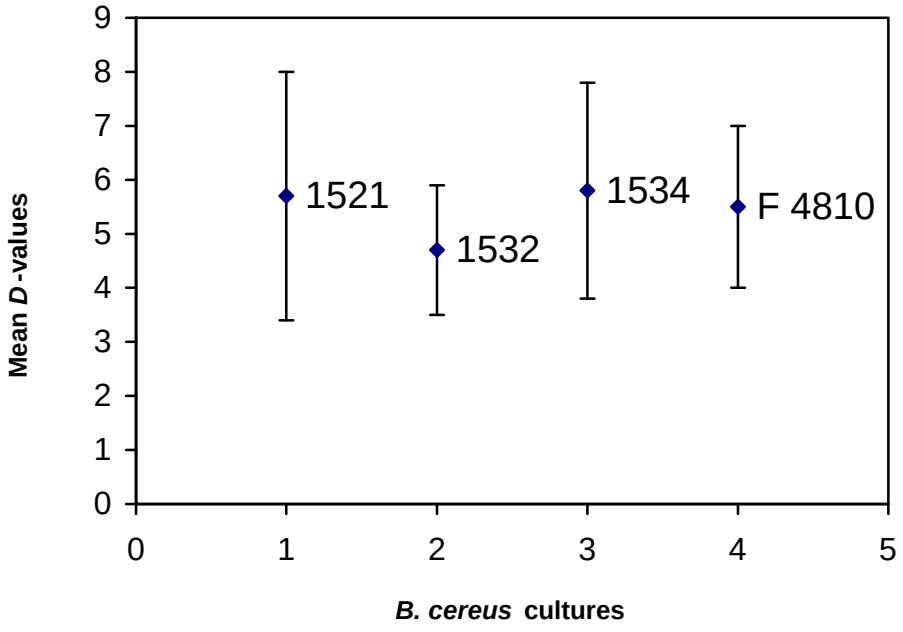


Figure 12 Thermal resistance patterns for vegetative cells of *B*. *cereus* cultures

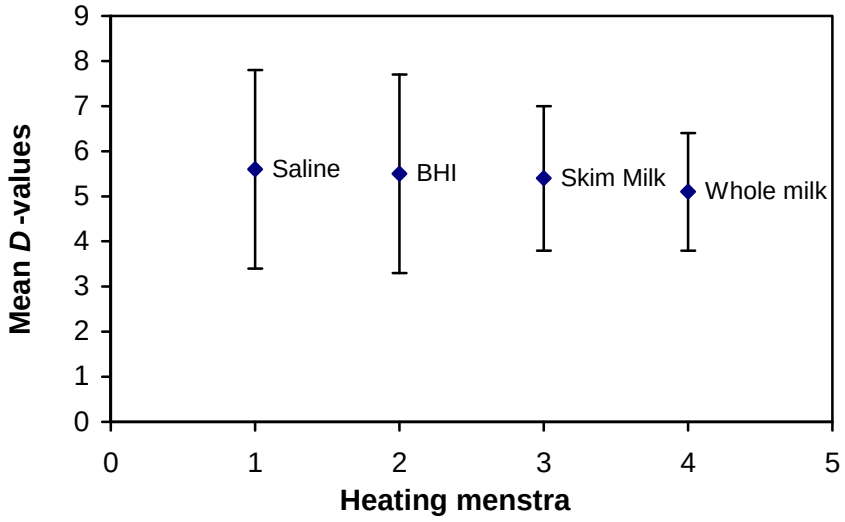


Figure 13 Thermal resistance patterns for vegetative cells of *B*. *cereus* cultures in different heating menstra

The mean *D*-values at 56, 58 and 60ºC across the cultures and heating media were 7.4, 4.9 and 3.8, respectively and was found to be statistically significant ($p > 0.05$) and thus indicating the sensitivity range of the cultures to temperature effect. Further, the inactivation phenomenon appears to be more pronounced at 58 to 60ºC, suggesting that 56ºC is too low a temperature to affect any significant inactivation of vegetative cells. The magnitude of decrease in *D*-values from D_{58} to D_{60} appeared to be low and a further increase in test temperature could have completely inactivated all the vegetative cells. A similarity to this could be evidenced in the study with vegetative cells of *C. perfringens* (an anaerobic sporeformer), wherein inactivation of cells occurred above 60°C. However, presence of fats and/or other food constituents in the medium could alter the heat resistance pattern (Doyle, 2002).

The findings observed with respect to thermal inactivation pattern of cultures studied become relevant when the projected commercial sterility values are presented and discussed. The 12 *D*-values, also termed as commercial sterility values for vegetative cells of *B*. *cereus* cultures ranged from a lowest of 42 min at 58 and 60ºC to a highest of 127.2 min at 56ºC (**Table 13**). The commercial sterility is defined as the condition in which no viable organism that exists can multiply under the storage conditions of foods. The 12 *D*-values for vegetative cells at a temperature of 60ºC across all the cultures and heating menstra was in the range of 42 to 51.6 min. This comes in close agreement with the general time-temperature combination of 62.8ºC for 30 min for the commercial low temperature long time holding (LTLT) method of pasteurization for fluid milk.

The *z*-values of vegetative cells of *B*. *cereus* cultures (**Table 14**) ranged from 9.3°C for *B*. *cereus* CFR 1521 in BHI broth to 24°C for *B*. *cereus* CFR 1532 in whole milk. The average *z*-values across the heating menstra recorded were 12°C for *B*. *cereus* CFR 1521, 18.5°C for *B*. *cereus* CFR 1532, 15.6°C for *B*. *cereus* CFR 1534 and

16.3°C for *B*. *cereus* F 4810. These values for different menstra tested for each of the cultures were not significantly different ($p < 0.05$), thus showing that the heating media did not influence as a singular effect on heat inactivation pattern of vegetative cells. Nevertheless, a progressive increase in z values was observed for most of the cultures in the ascending order of saline, BHI broth, skim milk and whole milk. The *z*-values recorded in the present study appear to be quite significant, as the heating menstra were not rich in concentration of major food constituents. Even in a study with pork luncheon roll, the *z*-value recorded for vegetative cells of *B*. *cereus* was 6.6°C (Byrne et al. 2006). On the same lines, the *z*-values could be compared with those of vegetative cells of *C. perfringens* in beef and turkey ranged from 6.22 to 6.77 °C (Juneja and Marmer, 1998).

Table 14. *z*-values for vegetative cells and spores of *B*. *cereus* cultures in different heating menstra

Culture	Heating menstra			
	Saline	BHI broth (Z value in °C)	Skim milk	Whole milk
		Vegetative cells		
B. *cereus* CFR 1521	12.1	9.3	12.5	14.2
B. *cereus* CFR 1532	12.5	16.1	21.2	24.0
B. *cereus* CFR 1534	10.3	14.3	16.7	21.0
B. *cereus* F 4810	13.8	14.3	16.0	21.0
		Spores		
B. *cereus* CFR 1521	25.0	25.0	33.3	33.3
B. *cereus* CFR 1532	20.0	25.0	25.0	33.3
B. *cereus* CFR 1534	16.6	20.0	25.0	38.4
B. *cereus* F 4810	16.7	26.3	29.4	33.3

The thermal death curve for the vegetative cells in the present study was non-linear (**Figure 11**), indicating that the inactivation rate was not constant, but rather exhibited a sigmoidal shape with shoulder and tailing pattern (Xiong et al. 1999). It is possible that the shoulder response could be due to clumping of the organisms in the suspension. Usually, all cells in the clump need to be inactivated prior to the colony forming ability of the clump could be destroyed and thus the lag phase appears in the form of shoulder (Adams and Moss, 1997). The effect could also be seen, if the inactivation phenomenon becomes cumulative rather than instantly lethal or if there are multiple target sites for inactivation (Xiong *et al*., 1999). If the lag phase represents a period in which the cells are able to re-synthesize a vital component, needed for repair or survival, death ensues only when the rate of destruction exceeds the rate of resynthesis (Mossel et al. 1995). The tailing pattern could be due to either one or combinations of the following like (i) presence of sub-populations of cells intrinsically more resistant than others. This is attributed to a factor called inherent biological variability. It denotes that though the cells are from the same clonal lineage they differ in their thermal sensitivity (Murphy et al. 1999), (ii) production of heat shock proteins (Pagan et al. 1997), (iii) protection by factors like dead cells, products of their destruction, and (iv) localized sites with low water activity and oxygen depletion in the heating medium.(Cerf, 1977). This could enable in survival under experimental conditions or reduction in the rate of inactivation process. The non-linear survival curve observed in this study could best be modelled by using modified Gompertz equation for determining thermal resistance (Bhaduri et al. 1991; Linton et al. 1995).

It is well known, that vegetative cells of *B*. *cereus* do not survive routine cooking temperatures, yet their study relating to thermal inactivation pattern holds significance due to the production of diarrhoeal and emetic toxins by these cultures during their vegetative phase. Besides, the ubiquitous presence of *B*. *cereus*, its ability to

contaminate a wide range of foods as pre- and post-processing contaminant and the increase in demand for minimally processed foods underscores the relevance of thermal inactivation of vegetative cells of *B*. *cereus*, which was hitherto not given due importance, except for an earlier study (Byrne et al. 2006).

4.2.3.2 Thermal inactivation of spores

Similar to the vegetative cells, the present study also included experimental trials for the evaluation of inactivation pattern of spores of *B*. *cereus* cultures in different heating menstra. The *D*-values recorded for spores of *B*. *cereus* cultures studied and the corresponding derived 12 *D*-values in different heating menstra are presented in **Table 15**. All the observations recorded were with R^2 (correlation coefficient) of 0.95 and the inactivation pattern was curvelinear (**Figure 14**). The *D*-values of spores ranged from a lowest of 4.4 min at 95°C to a highest of 19.45 min at 85ºC. These recorded values were very much in close agreement with those reported in an earlier study undertaken with a heating menstruum (pork luncheon roll), wherein the values were 29.5, 10.1 and 2.0 min at 85, 90 and 95ºC respectively (Byrne et al. 2006).

The thermal inactivation pattern of spores of *B*. *cereus* cultures observed in most of the earlier studies have revealed that *D*-values differ very much depending primarily on the strains used and to a lesser extent the extrinsic and intrinsic factors. Earlier studies have reported varying *D*-values for ATCC strains of *B*. *cereus* 4342, 7004 and 9818 in buffers and substrates similar to culture broth and those containing food constituents (Mazas et al. 1995, Mazas et al. 1999a; González et al. 1999; Montville et al. 2005). Moussa-Boudjemma et al. (2006) reported *D*-values of *B. cereus* strain ATCC 7004 as 6.7 at 90 °C in phosphate buffer (pH 7.0) which is a value close to that recorded in the present study for all strains studied across the different heating menstra. The same study concluded that acidification of the heating medium with acidulant, reduced

Table 15 Decimal reduction time* (*D*-values) of spores of *B*. *cereus* cultures

Culture	Heating menstra											
	Saline			BHI broth			Skim milk			Whole milk		
	85°C	90°C	95°C	85°C	90°C	95°C	85°C	90°C	95°C	85°C	90°C	95°C
	(Time, min)											
B. *cereus* CFR 1521	13.40[a] (160.8)	6.30[a] (75.6)	5.45[a] (66.0)+	12.50[ab] (150.0)	7.80[a] (93.6)	5.50[a] (62.4)	11.95[a] (144.0)	6.20[a] (74.4)	5.65[a] (68.4)	9.25[a] (111.6)	7.50[a] (90.0)	4.40[a] (52.8)
B. *cereus* CFR 1532	14.30[a] (171.6)	6.80[a] (81.6)	5.10[a] (61.2)	12.15[b] (145.2)	9.55[a] (114.0)	4.80[a] (57.6)	15.45[a] (186.0)	12.50[b] (150.0)	7.05[a] (84.0)	9.55[a] (115.2)	6.90[a] (81.6)	4.75[a] (57.6)
B. *cereus* CFR 1534	19.45[b] (234.0)	7.90[a] (94.8)	5.90[a] (70.8)	14.30[c] (171.6)	8.30[a] (99.6)	5.35[a] (63.6)	16.45[a] (198.0)	7.70[a] (92.4)	6.10[a] (73.2)	9.15[a] (110.4)	8.75[a] (105.6)	4.95[a] (60.0)
B. *cereus* F 4810	19.10[b] (229.2)	6.35[a] (76.8)	5.35[a] (64.8)	13.25[a] (159.6)	7.95[a] (96.0)	5.50[a] (67.2)	12.15[a] (146.4)	7.90[a] (94.8)	5.80[a] (69.6)	10.00[a] (120.0)	7.25[a] (87.6)	5.30[a] (69.6)
SEM	±1.00	±0.40	±0.31	±0.21	±0.35	±0.18	±1.26	±0.80	±0.53	±0.82	±0.47	±0.59

[a-c]Means of the same column followed by different letters differ significantly (P <0.05) according to Duncan's New Multiple Range Test

**D*-values are the means of two experimental trials, with each performed in duplicates

+Figures in parentheses indicate the 12D (commercial sterility) values

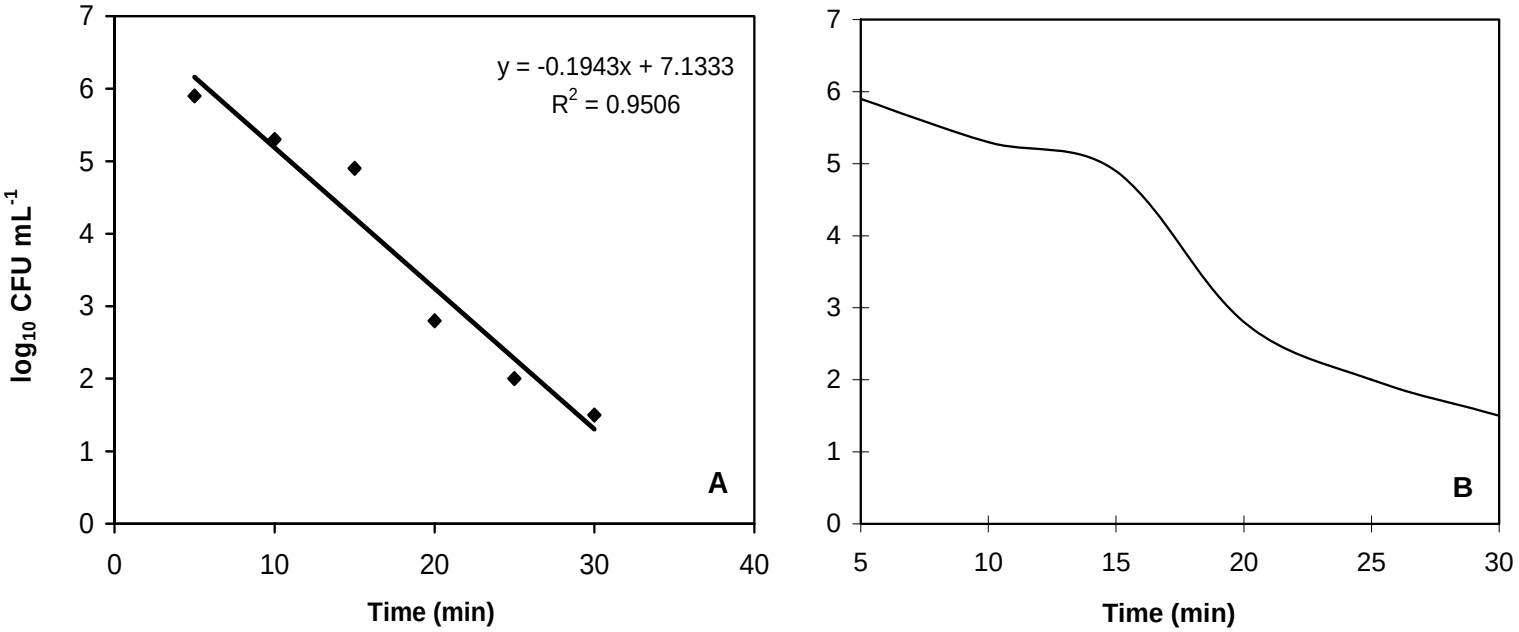


Figure 14 Survivor curves for spores of *B*. *cereus* CFR 1534 in skim milk at 95°C showing the line of best fit [**A**] and curvilinear curve [**B**]

the *D*-values of spores irrespective of the type of acidulant used. As pH decreased, the *D*-values decreased exponentially over the pH range (5.2 -4.0) yielding straight lines and basically parallel. This shows the effect of intrinsic factors like pH of the heating media to the thermal resistance of the organisms involved.

The *D*-values recorded in the present study for 3 cultures of *B*. *cereus* were almost closer to those values reported earlier for the strain of *B*. *cereus* ATCC 4342 and 7004 when subjected to heat treatment in buffers of pH 7.0 and milk medium at 90°C (Montville et al. 2005; Moussa-Boudjemma et al. 2006) and 95°C (Mazas et al. 1999a). In a study with spores of *B*. *cereus* ATCC 7004 (Mazas et al. 1999b), the *D*-values recorded at 92 and 96°C in saline and milk medium were quite lower to those observed in this study. As *B*. *anthracis* was in the same cluster as that of *B*. *cereus*, a similarity was observed in an earlier study with spores of *B*. *anthracis* Sterne (Novak et al. 2005), wherein the *D*-values recorded at 100°C in skim milk was almost near to those recorded in the present study. In contrast to the above, studies with different cultures like that of *B*. *anthracis* in whole milk at 100°C (Xu et al. 2006) and a strain of *B*. *cereus* ATCC 9818 at 100°C in distilled water, BHI broth and skim milk (Novak et al. 2005) revealed higher *D*-values (14.4 to 35.7 min) in comparison to those recorded in this study with 3 native food isolates of *B*. *cereus*.

The wide variations observed in the thermal inactivation pattern of spores of *B*. *cereus* could be attributed to one or more of the following like (i) characteristics of strains of *B*. *cereus* with environmentally induced resistance due to dipicolinic acid content, degree of hydration and mineral content (Marquis and Shin, 1994; Palop et al. 1996; Mazas et al. 1999b; Melly et al. 2002; Baweja et al. 2008), (ii) temperatures which have induced spore formation as well as other conditions prevailing during sporulation (Condon et al. 1992; Baweja et al. 2008) and (iii) heterogeneity in the cluster of spores with respect to germination and/or survival (Byrne et al. 2006).

Similar to the behavioural pattern of vegetative cells, even the spores of *B*. *cereus* cultures used in the present study showed certain differences in their thermal resistance. The mean *D*-values obtained for cultures of *B*. *cereus* CFR 1521, 1532 and 1534 are presented in **Figure 15**.The isolate of *B*. *cereus* CFR 1521 exhibited a lowest mean *D*-value of 8 min, whereas the potent toxigenic isolate of *B*. *cereus* CFR 1534 had a mean *D*-value of 9.5 min. Further, mean *D*-values obtained for the spores in different heating menstra like saline, BHI broth and skim milk were 9.6, 8.9 and 9.6 min, respectively (**Figure 16**). The lowest of 7.3 min was observed with whole milk, which goes to indicate that spores present in this medium were more sensitive to heat treatment. The mean *D*-values in the heating menstra tested for each of the isolates were not significantly different (P <0.05), wherein it could be inferred that medium composition of heating menstra did not appear to have any marked influencing effect on the inactivation profile of the cultures studied.

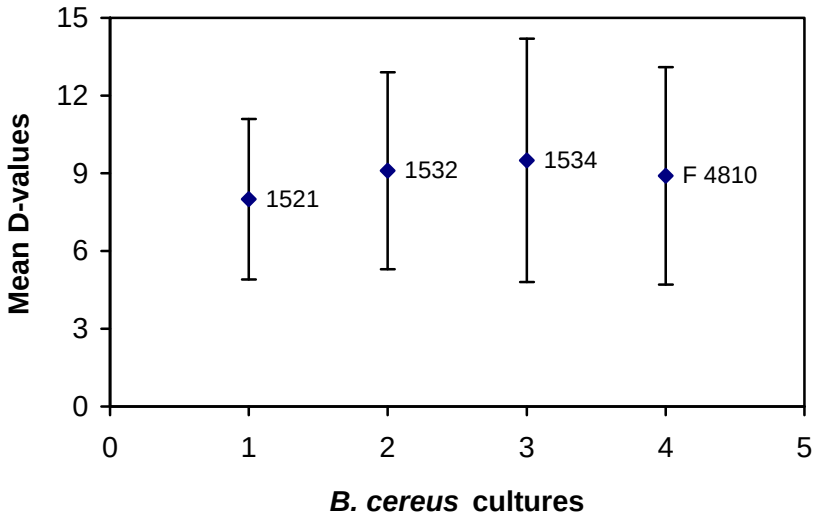


Figure 15 Thermal resistance patterns for spores of *B*. *cereus* cultures

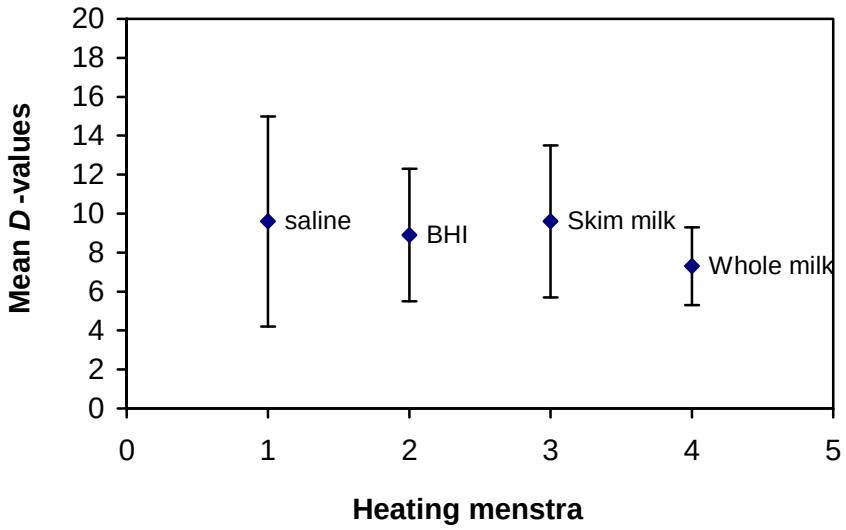


Figure 16 Thermal resistance patterns for spores of *B*. *cereus* cultures in different heating menstra

The survival curves obtained for spores of isolates of *B*. cereus tested in the present study showed a curvilinear pattern with shoulder (lag phase) followed by a linear declining pattern, an observation reported in one of the earlier studies (Kamau et al. 1990). Like-wise, Loss and Hotchkiss (2002) showed that the thermal inactivation of bacteria does not always follow first order reaction kinetics, indicating that the death rate was not constant throughout the heat treatment period.

The shoulder effect could be due to clumping of spores, which could result in an increase of thermal resistance. Hence, all spores in the clumps need to be inactivated prior to the destruction of colony forming ability of the clump (Adams and Moss, 1997; Furukawa et al. 2005). The effect could also be due to the heterogeneous sub-populations of spores (dormant, germinated and inactivated) differing in their physiological state during heat treatment and become resistant within a highly extreme resistant sub-population (Peleg and Cole, 1998). The lag phase in spore inactivation

pattern reveals that there was a small decrease in the concentration of spores and the linear decline phase shows that specific death rate was constant. This follows the first order kinetics and goes to indicate that as the microbial population is subjected to heat treatment, the spores get inactivated at a constant rate. The absence of any drastic decrease in the initial phase of inactivation rules out the possibility of vegetative cells being present in the spore suspension used in this study.

The z-values of the spores of isolates of *B. cereus* ranged from 16.6 to 38.4°C (**Table 14**). The average z-values (°C) for individual isolates across the menstra were 29.2 ± 4.8 for *B*. *cereus* CFR 1521, 25.8 ± 5.5 for CFR 1532, 25 ± 9.5 for CFR 1534 and 26.4 ± 7.0 for *B*. *cereus* F 4810. The z-values recorded in the study can be compared to an earlier study on spores of *B. anthracis* Sterne and *B. cereus* ATCC 9818 in similar heating media (distilled water, BHI broth and skim milk) with z-values ranging from 20.4 to 36.5ºC (Novak et al. 2005).

Earlier documented reports in the literature have shown that high level of fat (>20%) provides a protective effect for bacterial pathogens subjected to heat treatment (Fain et al. 1991; Murphy et al. 2004). This protective effect could be a consequence of reduced water activity in milk medium (Senhaji and Loncin, 1977). In the present study, the fat level in whole milk was 3% and negligible in skim milk, wherein there may not be very significant protective effect of lipids for the spores of *B*. *cereus*. Mazas et al. (1999a) reported a similar phenomenon wherein, the *z*-values were not significantly modified by milk composition in a study involving spores of *B. cereus* strains ATCC 7004, 4342 and 9818 and the milk composition did not have any effect on the thermal resistance. Similarly, Xu et al. (2006) indicated that the thermal resistance of spores of *B*. *anthracis* in whole milk was not different from that of skim milk.

It is well established, that pasteurization process kills vegetative cells. However, spores, which are formed from the same vegetative cells, achieve resistance to heat

processing. This resistance is invariably attributed to a metabolically inactive complex of nucleic acids and calcium dipicolinic acid formed under a set of prevailing unfavourable conditions (Baweja et al. 2008). Besides these, several extrinsic factors play a role in the determination of heat resistance of the organisms. Loss and Hotchkiss (2002) reported that the dissolved carbon dioxide concentration decreased the *D*-values of both the phases namely the vegetative cells of *Pseudomonas fluorescens* and spores of *B. cereus* ATCC 14579 in milk which shows that the thermal sensitivity of the organisms is dependent on the gases dissolved in the heating medium. Penna and Moraes (2002) and Wandling et al. (1999) demonstrated that antibiotics like nisin could reduce the thermal resistance of the *B. cereus* strains. In a study involving spores of *B. cereus* ATCC 14798 in milk supplemented with nisin, the apparent *D*-values decreased by 40% over a test temperature range of 80 to 100°C. Gonzalez et al. (1997) and Mazas et al. (1999b) reported that the presence of NaCl concentration increased the heat resistance of spores of *B. cereus* (strains ATCC 7004, 4302 and 9818), although the effect varied among the strains. Monoglycerides are known to enhance the thermal sensitivity of *B. cereus* and thus can be used to reduce the thermal processes required to inactivate the bacterial spores (Chaibi et al. 1998).

Mazas et al. (1995) observed that sporulation media had a role to play in determining the thermal resistance of the strains tested and use of a single sporulation medium to obtain maximum heat resistance is not recommended. The temperature of sporulation is known to affect the heat resistance of the spores. Generally, a higher heat resistance is observed for spores sporulated at high temperatures. Condon et al. (1992) concluded from their studies on *B. subtilis* that greater heat resistance at higher temperatures of sporulation was not due to selection of more heat resistant cells by a higher sporulation temperature, but it was due to the ability of the cells to inherit the

nature of sporulation at high range of temperature and their final heat resistance was determined by their temperature of sporulation.

Studies by Dumalisile et al. (2005) showed that thermal inactivation involving *B. cereus* in milk with Low Temperature Long Time (LTLT) and High Temperature Short Time (HTST) resulted in *B. cereus* emerging as lone survivor amongst the other background microflora. Further, in the same study, post-pasteurization resulted in the survival of *B. cereus* along with few other microflora indicating that post-pasteurization is not an effective method to inactivate the organism. In general, as the pasteurization temperature is increased, exposure times are decreased to prevent excessive degradation of milk protein. In this regard, Novak et al. (2005) studied thermal inactivation of spores of *B. cereus* ATCC 9818 and *B. anthracis* in different heating media (distilled water, BHI and skim milk) at 72 and 78°C to evaluate standard milk pasteurization parameters and 100, 130 and 150°C to simulate ultra high temperature processing. It was observed that at 72 and 78°C, heat treatment even beyond recommended times of 15 to 30 seconds were not effective in inactivating the spores of the strains. This showed that higher temperature and longer time are needed to inactivate the spores of the strains.

In the Indian context, a study by Pedurkar and Kulkarni (1990) reported higher *D*-values in the range of 19 to 54 min at 100°C for *B. cereus* and *B. licheniformis*, respectively, in milk and rice medium. An interesting observation from the study was none of the *Bacillus* species was completely inactivated when added to rice and cooked for 30 min.

In the background of findings of present study and few other earlier reports, it would be difficult to propose a baseline thermal processing schedule, which would be effective against spores of other related genera and/or species. This observation is presented after analyzing earlier investigations on *z*-values recorded for spores of *C*.

perfringens in food matrix and *C*. *sporogenes* in phosphate buffer (Byrne et al. 2006; Mah et al. 2008). However, based on the z-values recorded in the present study (**Table 14**), it could be inferred that in all probability, spores of very closely related bacterial species can become inactive.

The research leads from present study which included control and representative heating media gave an insight about the thermal inactivation pattern of native food isolates of *B*. *cereus* and provided baseline data to formulate time-temperature combinations during thermal processing of high complex food matrices. Although, strains of different species of a genus arise from a single origin, it is very difficult to conclusively infer that single time-temperature treatment would be effective in bringing about their inactivation. Considering the genetic make-up and the prevailing habitat, it becomes important to generate data for at least commonly encountered cultures of public health significance in a few heating menstra. This understanding of thermal resistance of spores and vegetative cells would help in modelling the combined effects for inactivation and assessing the benefits of optimum time-temperature combined unit operations to achieve microbiologically safe food for human consumption in the food chain of traditional foods.

The new and changing regulatory parameters not only create greater opportunities for customized process, but also it puts significant pressure on the industry to comply with the standards. In this backdrop, the thermal inactivation parameters and time-temperature combinations documented in literature from laboratory findings come in handy for the processors, especially for those engaged in continual development of new products and processes and help in future risk assessment (Marks, 2001).

The results revealed varying and high *D*-values for vegetative cells and spores of native food isolates of *B*. *cereus* tested in selected heating menstra. The z-values reported in this study for spores of *B*. cereus appear to be higher than those values

reported in earlier studies. Hence, a combination of poor hygienic practices and insufficient heat treatment could result in microbiologically poor quality of foods and thereby pose potential threat in the form of health hazards. While thermal processing remains the primary method for both, value addition and ensuring microbial safety, many technologies like irradiation, ultra high pressure, pulsed electric fields and others are becoming an integral part of food processing industry. Nevertheless, thermal processing will continue to be a prominent method to impart desirable characters and economic value to foods.

4.3 INFLUENCE OF CULTURAL AND NUTRITIONAL ATTRIBUTES ON THE BEHAVIOURAL PATTERN OF *BACILLUS CEREUS* (VEGETATIVE CELLS AND SPORES) IN BROTH SYSTEM

The growth of spoilage or pathogenic microorganisms in foods is of serious public health concern. Microbial population in final food product depends on the initial level of bacterial contamination as well as intrinsic and extrinsic attributes like storage temperature, pH, water activity, preservatives, additives and others, which together may influence survival or growth or inactivation. The number of variables affecting the growth kinetics of an organism is large, however, few factors such as incubation temperature, pH, salt concentration, water activity, etc., are predominant factors that affect the growth of the organisms. Predictive Modelling deals with the incorporation of mathematical models to define growth kinetics of microorganisms in foods. Modelling of microbial growth response allows the development of decision support system of microbial spoilage / risks involved through foods.

Predictive models for growth kinetics have been generally centered on logarithmic phase of microbial growth in foods and broth systems. However, it is the lag phase in microbial growth pattern, which is to be considered as more relevant. Lag phase duration (LPD) is the period following the inoculation of microorganisms into fresh culture medium when there is no increase in cell numbers or mass. It is related to the time required for the cells to adjust physiologically to a new environment. Growth rate (GR) is another important kinetic parameter which is described as the number of generations per unit time. LPD and GR are the two prominent indices which define the behaviour of a given organism within a defined set of intrinsic and extrinsic factors.

In the present experimental trials, an attempt was made to model the behaviour of vegetative cells and spores of a potent toxigenic native food isolate of *B*. *cereus* CFR

1534 in terms of LPD and GR as a function of temperature, pH and sodium chloride concentration in broth system.

4.3.1 MATERIALS

All glasswares, media and other materials used in the present study were either wet sterilized or dry sterilized. Wet sterilization was carried out at 121°C for 20 min in an autoclave and dry sterilization at 180°C for 4 h in a Hot Air Oven. All bacteriological media used were those of dehydrated media procured from Hi-Media Lab., Mumbai, India (Hi-Media, 2003). The media were prepared as per manufacturer's instructions. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

4.3.1.1 Bacterial (test) culture

This included a native food isolate of *B*. *cereus* CFR 1534 (**Table 2 under 4.1.3.1; pp 99**). The culture was maintained at 6°C on brain heart infusion (BHI) agar slants and propagated twice successively in BHI broth at 37°C prior to use in experimental trials.

4.3.1.2 Diluent

The diluent used was 0.85% normal saline as described earlier under **4.1.1.3.1** (**pp 76**).

4.3.1.3 Bacteriological media

4.3.1.3.1 Brain heart infusion (BHI) broth and agar

This was prepared as described earlier under **4.1.1.7.3** (**pp 78**) and used in this study.

4.3.1.3.3 Nutrient broth and agar

This was prepared as described earlier under **4.1.1.7.7** (**pp 80**) and used in this study.

4.3.2 METHODOLOGY

4.3.2.1 Inoculum preparation of vegetative cells and spores of test culture

The requisite suspension of vegetative cells and spores was prepared as described earlier under **4.2.2.2** (**pp 135**) and **4.2.2.3** (**pp 136**), respectively.

4.3.2.2 Experimental design

Taking into consideration the literature information about the growth behaviour of *B*. *cereus* in laboratory media under influencing factors (Goepfert et al. 1972), preliminary experimental trials were undertaken to assess the upper and lower range of values for the 3 factors namely incubation temperature, pH level and sodium chloride concentration. This was ascertained by visualizing initiation of growth of *B*. *cereus* CFR 1534 (turbidity of broth medium), when introduced at an initial inoculum of 3.3 $\log_{10}$ CFU/ml in laboratory growth medium. This experimental trial was very important so as to avoid any 'no growth condition' in the experimental design.

The experimental design was a central composite design (CCD) based on 3 factors and 5 levels. The factors for vegetative cells were incubation temperature in the range of 12 to 48°C, pH level of 5.5 to 7.5 and sodium chloride concentration of 2 to 6%. Similarly, in the case of spores, the ranges of pH and sodium chloride levels were the same, while incubation temperature range was 22 to 42°C. The CCD based on the above factors and variables resulted in 21 combinations for this experimental design, which is shown **Table 16**.

Multiple tubes of BHI broth in 10 ml aliquots with requisite levels of NaCl and pH as per the experimental design were prepared and sterilized. The individual test tubes were inoculated with aliquots 0.1 ml inoculum (vegetative cells and spores in separate sets) to give a final concentration of 3.3 $\log_{10}$ CFU/ml and incubated at desired temperatures. Experimental samples of culture broths were enumerated for the viable

Table 16 Experimental design (CCD) for vegetative cells and spores of *B*. *cereus*

Vegetative cells			Spores		
Temp (°C)	pH level	NaCl conc.	Temp (°C)	pH level	NaCl conc.
19.3	5.9	2.8	26.1	5.9	2.8
40.7	5.9	2.8	37.9	5.9	2.8
19.3	7.1	2.8	26.1	7.1	2.8
40.7	7.1	2.8	37.9	7.1	2.8
19.3	5.9	5.2	26.1	5.9	5.2
40.7	5.9	5.2	37.9	5.9	5.2
19.3	7.1	5.2	26.1	7.1	5.2
40.7	7.1	5.2	37.9	7.1	5.2
12.0	6.5	4.0	22.0	6.5	4.0
48.0	6.5	4.0	42.0	6.5	4.0
30.0	5.5	4.0	32.0	5.5	4.0
30.0	7.5	4.0	32.0	7.5	4.0
30.0	6.5	2.0	32.0	6.5	2.0
30.0	6.5	6.0	32.0	6.5	6.0
30.0	6.5	4.0	32.0	6.5	4.0
30.0	6.5	4.0	32.0	6.5	4.0
30.0	6.5	4.0	32.0	6.5	4.0
30.0	6.5	4.0	32.0	6.5	4.0
30.0	6.5	4.0	32.0	6.5	4.0
30.0	6.5	4.0	32.0	6.5	4.0

counts of inoculated *B*. *cereus* for the respective set of parameters by removing the test tubes periodically from incubation and surface plating of 0.1 ml aliquots of appropriate dilutions on pre-poured plates of nutrient agar in duplicates. The inoculated plates were incubated for 24-48 h at 37°C. Characteristic colonies of *B. cereus* appearing in the incubated plates were counted and expressed as average $\log_{10}$ CFU/g. The experimental trials were carried out independently, in duplicates and the mean values with statistical analysis were considered in presenting the data.

4.3.2.3 Determination of lag phase duration and growth rate

The derived viable populations (log_{10} CFU/ml) of *B. cereus* were used to calculate lag phase and growth rate with DMFit software programme version 2.0 (Institute of Food Research, Norwich, UK) as a function of Baranyi model (Baranyi and Roberts, 1994) for vegetative cells and spores of *B*. *cereus*, individually.

4.3.2.4 Multivariate analysis and response surface plots

The generated experimental data in the individual sets of vegetative cells and spores were subjected to multiple regression analysis (Microsoft Excel Software Programme (version 8.0, 2003, Microsoft Corporation, Redmond, WA, USA) to derive equations for individual parameters of LPD and GR, respectively, which was further expressed as a quadratic function of incubation temperature, pH level and NaCl concentration using the following equation:

$$Prd = x_1 + x_2T + x_3P + x_4N + x_5T^2 + x_6T*P + x_7P^2 + x_8P*N + x_9N^2 + x_{10}T*N$$

where Prd is the predicted value for LPD and GR; x_n (n = 1, 2, 3……..10) are the coefficients; T is the incubation temperature (°C); P is the pH level; and N is the concentration of NaCl (%).

Statistical testing of the model was performed using analysis of variance (ANOVA), which was used to test the significance and adequacy of the model. The coefficient of determination (R^2) value near to 1.0 indicates a high degree of concentration between observed and predicted values. Statistical significance was determined based on Fischer *F* test resulting in *F* value of $P > 0.05$. The value of significance of *F* less than 0.05 show that model terms are significant, whereas values greater than 0.10 indicate no significance. The coefficient values were calculated and tested for significance. Those with very small *P* values ($P < 0.05$) were considered significant and others as not significant. The R^2 value obtained shows the effectiveness

of equation to derive model fits for predicting the growth behavior of *B. cereus* in terms of LPD and GR. The feasibility to predict growth responses depends on these statistical indices (Duffy et al.1994).

Three dimensional response surface plots were generated to depict the interaction of the dependent and independent variables. The effects of independent variables on LPD and GR were evaluated using these three-dimensional plots obtained by imposing a constant value to one variable at a time.

4.3.3 RESULTS AND DISCUSSION

4.3.3.1 Lag phase duration in vegetative cells of *B. cereus*

The data obtained from the experimental trials was subjected to the model of Baranyi and Roberts (1994), wherein curves were fitted with $\log_{10}$ CFU/ml counts against time at each combination of factors and LPD values derived. The experimental LPD values were subjected to multiple regression analysis and the derived predicted values are presented in **Table 17**. The experimental LPD values ranged from 3.1 to 31.5 h. The lowest LPD was recorded under defined conditions of 30°C, pH of 5.5 and NaCl concentration of 4%. Similarly, the highest value was recorded at conditions of 48°C, pH of 6.5 and NaCl concentration of 4%. The derived regression coefficients, their standard errors and *P* values are detailed in **Table 18**. The R^2 value of 0.78 indicates a fairly high degree of correlation between observed and predicted values. The linear coefficient temperature alone was significant with a very small *P* value ($P < 0.05$) and the other term coefficients were not statistically significant. It could be visualized that incubation temperature primarily influenced LPD of vegetative cells of *B*. *cereus*.

Earlier research investigations on similar lines with determination of growth kinetic parameters as a function of temperature, pH, sodium chloride and sodium nitrite observed LPD value as 16.86 h under defined set conditions of 19°C, pH of 2.5

Table 17 Experimental and predicted LPD values of vegetative cells of *B. cereus* CFR 1534

Treatment number	Temperature (°C)	pH Level	NaCl conc. (%)	Experimental LPD (h) ± SD	Predicted LPD (h)
1	19.3	5.9	2.8	21.0 ± 7.1	18.4
2	40.7	5.9	2.8	12.1 ± 0.1	13.0
3	19.3	7.1	2.8	17.3 ± 6.4	14.8
4	40.7	7.1	2.8	5.8 ± 0.4	9.5
5	19.3	5.9	5.2	19.2 ± 1.0	16.4
6	40.7	5.9	5.2	6.9 ± 2.5	10.2
7	19.3	7.1	5.2	17.0 ± 2.2	17.0
8	40.7	7.1	5.2	7.4 ± 1.2	10.9
9	12.0	6.5	4.0	29.9 ± 1.4	35.0
10	48.0	6.5	4.0	31.5 ± 1.6	25.3
11	30.0	5.5	4.0	3.1 ± 0.1	4.3
12	30.0	7.5	4.0	4.2 ± 1.7	1.9
13	30.0	6.5	2.0	6.6 ± 1.6	7.4
14	30.0	6.5	6.0	8.7 ± 0.8	6.8
15	30.0	6.5	4.0	4.4 ± 0.2	7.7
16	30.0	6.5	4.0	6.7 ± 1.7	7.7
17	30.0	6.5	4.0	7.0 ± 2.3	7.7
18	30.0	6.5	4.0	7.7 ± 2.2	7.7
19	30.0	6.5	4.0	8.8 ± 0.6	7.7
20	30.0	6.5	4.0	9.7 ± 0.3	7.7
21	30.0	6.5	4.0	9.4 ± 2.3	7.7

Table 18 Coefficients and associated standard error derived by multivariate analysis for LPD of vegetative cells of *B*. *cereus* CFR 1534

Source	Coefficient	SE	*P*
Intercept	-75.0	132.6	0.58
T	-4.4	1.5	0.01
P	53.1	36.6	0.17
N	-7.9	13.5	0.57
T^2	0.1	0.01	5.01E
T x P	0.0	0.2	0.98
P^2	-4.6	2.7	0.11
P x N	1.4	1.8	0.44
N^2	-0.2	0.7	0.82
N x T	-0.01	0.1	0.89
R^2 0.78			

T, incubation temperature (°C); P, pH level; N, NaCl concentration; R^2, Coefficient of determination

and NaCl concentration of 2.5% (Benedict et al. 1993). Most of the values reported in their study correlate with those obtained in the present experimental trials under similar conditions for growth of vegetative cells. Peters et al. (1991) studied the effect of salt concentration on growth of *B.* cereus with continuous gradients of pH and temperature. These researchers noted that at NaCl concentration of 0.5%, growth occurred over the entire temperature range examined (14 to 41°C) with a minimum pH of 4.7. When the salt concentration was increased to 3 and 5%, the temperature range wherein the growth was observed decreased slightly by 1°C on either side in the range with minimum

pH levels of 4.9 and 5.5, respectively. No growth was observed at 7% NaCl level. It could be seen that a similar pattern of growth accompanied by varying LPD was observed in the present experimental trials.

Valik et al. (2003) determined LPD values for *B. cereus* and other microflora in pasteurized milk. The values for *B. cereus* were found high enough to allow the background flora to grow to a population level of 4 $\log_{10}$ CFU/ml in pasteurized milk stored at low temperatures (5 to 13°C), before *B. cereus* could reach the risk population level. This shows the significance of LPD, wherein the background microflora tend to over grow the risk causing bacterial species (like *B*. *cereus* in this case) and delay the onset of risk causing hazards in foods.

Although deviations did exist when the present experimental (observed) LPD values were compared with an established Pathogen Modelling Program (PMP), the value of 3.1 h obtained at a combination factors of 30°C, pH 5.5 and NaCl concentration of 4% was quite near to the predicted value of 3.9 h by PMP. This indicates that the data obtained in the present study has the potentiality to develop predictive models for risk causing hazards through foods at certain combinations of factors.

The coefficients derived by multivariate analysis were utilized to generate response surface plots for LPD values of vegetative cells of *B*. *cereus* CFR 1534 as a function of varying incubation temperatures and NaCl concentrations at 3 defined pH levels of 5.5, 6.5 and 7.5. The response surface model for LPD was proposed to be:

$$LPD = -75.0149 - 4.39377T + 53.12556P - 7.88665N + 0.069246T^2 + 0.004089T*P - 4.63506P^2 + 1.451389P*N - 0.15627N^2 - 0.01451N*T$$

where LPD is the lag phase duration; T is the incubation temperature; P is the pH level; and N is the sodium chloride concentration.

The individual response surface plots at the defined pH levels are shown in **Figure 17**.

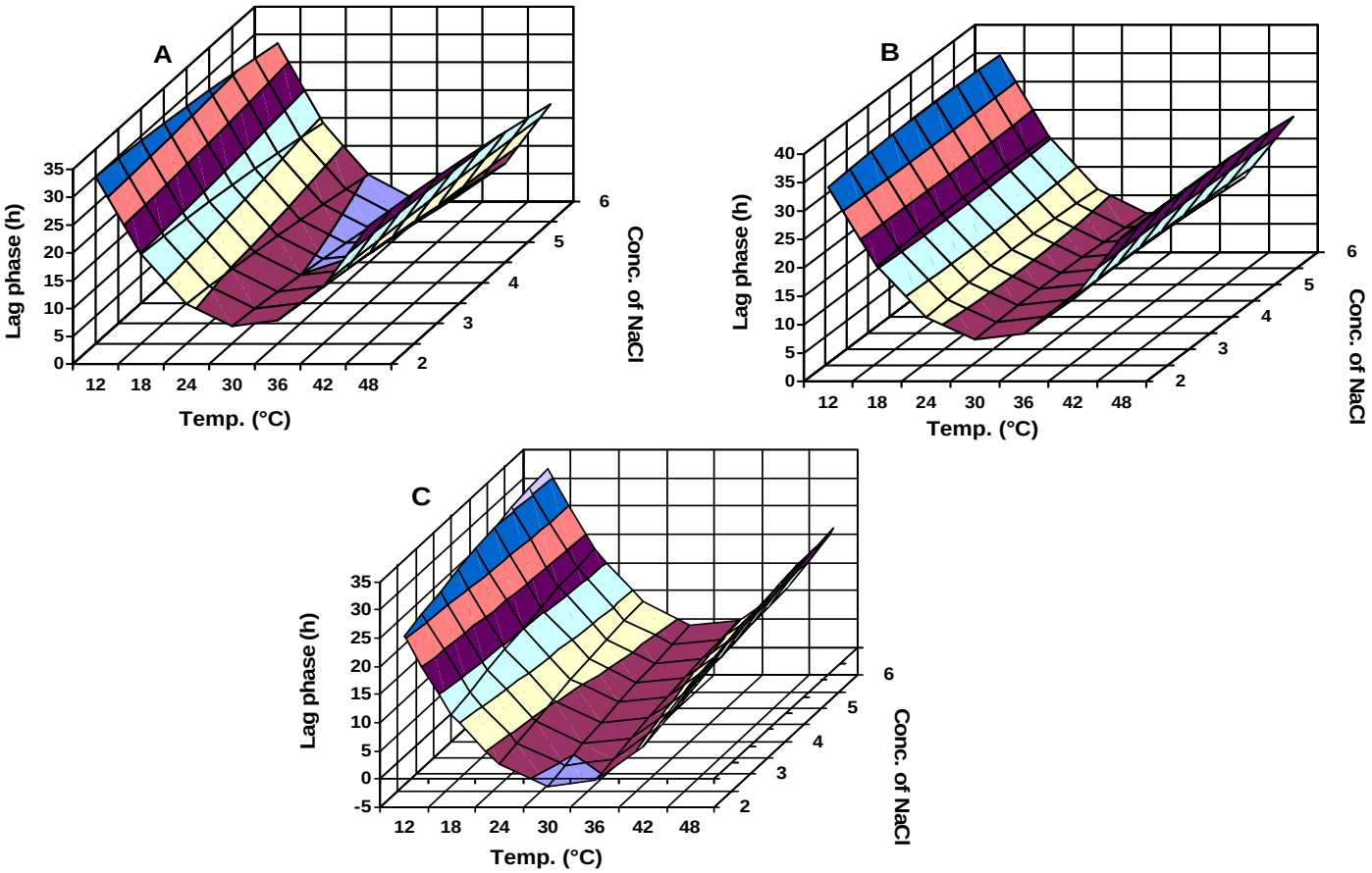


Figure 17 Response surface plots for LPD of vegetative cells of *B. cereus* CFR 1534 grown in BHI broth at pH levels of 5.5 [**A**], 6.5 [**B**] and 7.5 [**C**] $\log_{10}$ CFU/ml, respectively

Among the range of LPD values generated, at pH levels of 5.5 and 6.5, lower LPD values were observed with incubation temperatures of 30 and 36°C. At these two temperatures, the LPD values progressively decreased with increasing concentrations of NaCl from 2 to 6%, which was quite drastic at pH 5.5 (**Figure 17 A** and **B**). At the lowest and highest incubation temperatures used in this experimental design, the LPD values were in higher range and decrease in their values over increasing NaCl concentrations was not very appreciable. In contrast, at pH 7.5 with incubation temperatures of 30 and 36°C, the LPD values were very low with negative values being observed initially at NaCl concentrations of 2 and 2.5%. However, with increasing NaCl concentration, the LPD values revealed an increasing trend, with higher values at temperatures of 12 and 48°C and lower values at 24 to 36°C (**Figure 17C**).

If one considers LPD of bacterial organisms as a potential marker of microbial safety in the food chain, it is of interest to visualize from the generated response surface plots that selected storage temperatures and salt levels in foods can provide unfavourable conditions for an opportunistic pathogen like *B*. *cereus* to cause health associated risks.

4.3.3.2 Growth rate of vegetative cells of *B. cereus*

Similar to the derivation of LPD values, growth rate (GR) for *B*. *cereus* was obtained through the use of model of Baranyi and Roberts (1994), with the experimental values being derived at each combination of factors. The experimental GR values were subjected to multiple regression analysis and the derived predicted values are presented in **Table 19**. The experimental GR values ranged from 0.2 to 2.2/h. The lowest GR of 0.2/h was recorded at a combination of 48°C, pH 6.5 and NaCl concentration of 4%, whereas the highest GR of 2.2 was with a combination of 30°C, pH 7.5 and 4% NaCl.

Table 19 Experimental and predicted GR values of vegetative cells of *B. cereus* CFR 1534

Treatment number	Temperature (°C)	pH Level	NaCl conc. (%)	Experimental GR (value/h) ± SD	Predicted GR (value/h)
1	19.3	5.9	2.8	1.3 ± 0.5	1.3
2	40.7	5.9	2.8	1.4 ± 0.6	1.3
3	19.3	7.1	2.8	1.0 ± 0.9	1.5
4	40.7	7.1	2.8	1.4 ± 0.3	1.3
5	19.3	5.9	5.2	0.7 ± 0.5	1.0
6	40.7	5.9	5.2	0.9 ± 0.4	0.7
7	19.3	7.1	5.2	1.2 ± 0.3	1.5
8	40.7	7.1	5.2	0.9 ± 0.1	1.0
9	12.0	6.5	4.0	1.5 ± 0.1	1.0
10	48.0	6.5	4.0	0.2 ± 0.0	0.5
11	30.0	5.5	4.0	1.3 ± 0.1	1.4
12	30.0	7.5	4.0	2.2 ± 0.0	1.8
13	30.0	6.5	2.0	1.4 ± 0.1	1.4
14	30.0	6.5	6.0	1.2 ± 0.4	1.0
15	30.0	6.5	4.0	1.6 ± 0.2	0.9
16	30.0	6.5	4.0	0.9 ± 0.1	0.9
17	30.0	6.5	4.0	0.6 ± 0.1	0.9
18	30.0	6.5	4.0	0.4 ± 0.2	0.9
19	30.0	6.5	4.0	0.9 ± 0.2	0.9
20	30.0	6.5	4.0	0.9 ± 0.3	0.9
21	30.0	6.5	4.0	0.9 ± 0.2	0.9

The derived regression coefficients, their standard errors and *P* values are detailed in **Table 20**. The R^2 value of 0.49 indicates a poor correlation between the observed and predicted values. The linear coefficients of pH and cross product coefficient (pH x pH) were significant with very small *P* values (*P* <0.05). The other term coefficients were not statistically significant. It could be visualized that pH level was of major influence on the test culture of *B*. *cereus* CFR 1534.

Table 20 Coefficients and associated standard error derived by multivariate analysis for GR of vegetative cells of *B*. *cereus* CFR 1534

Source	Coefficient	SE	*P*
Intercept	32.1	15.0	0.05
T	0.1	0.2	0.67
P	-9.2	4.1	0.05
N	-1.2	1.5	0.43
T^2	0.0	0.0	0.62
T x P	-0.0	0.0	0.82
P^2	0.7	0.3	0.04
P x N	0.1	0.2	0.59
N^2	0.1	0.1	0.38
N x T	-0.0	0.0	0.65
R^2	0.49		

T, incubation temperature (°C); P, pH level; N, NaCl concentration; R^2, Coefficient of determination

In the absence of many studies focusing on the factors which tend to influence growth profile of *B*. *cereus*, a recent study (Heo et al. 2009) with an emetic strain of *B.* cereus F4810/72 revealed GR values in the range of 0.01 to 0.90/h in Tryptic soy broth as a function of temperature (10 to 40°C) pH (5.5 to 8.5) and NaCl concentration (0 to 8%). The primary model they worked showed a good fit to the Gompertz equation used to obtain growth rate. In the present study, the growth rate values obtained were in the range of 0.2 to 2.2/h, which almost comes very near to those reported using Gompertz function. Although the model derived for GR of *B*. *cereus* was not of high accuracy, to a certain extent, a moderate level of agreement could be compared with the PMP, wherein the predicted GR value of 0.41/h at a combination of 30°C, pH 5.5 and 4% NaCl concentration was 1.3/h (observed value) in the present study.

The coefficients derived by multivariate analysis were utilized to generate response surface plots for GR of vegetative cells of *B*. *cereus* CFR 1534 as a function of varying incubation temperatures and NaCl concentrations at 3 defined pH levels of 5.5, 6.5 and 7.5. The response surface model for GR was proposed to be:

$$GR = 32.08534 + 0.073552T - 9.24864P - 1.25244N - 0.00048T^2 - 0.00545T*P + 0.704837P^2 + 0.114583P*N + 0.069959N^2 - 0.00535N*T$$

where GR is the growth rate; T is the incubation temperature; P is the pH level; and N is the sodium chloride concentration.

The individual response surface plots at the defined pH levels are shown in **Figure 18**.

In contrast to the response surface plots generated for LPD values of *B*. *cereus*, the plots derived for GR values appear to have a varied pattern at the three pH levels of 5.5, 6.5 and 7.5 included in the present study. At pH 5.5, GR values of less than 1.0/h was observed at (i) an incubation temperature of 42°C and NaCl concentration of 5.5 and 6% and (ii) 48°C with NaCl levels of 4.5, 5, 5.5 and 6% (**Figure 18A**). At all other

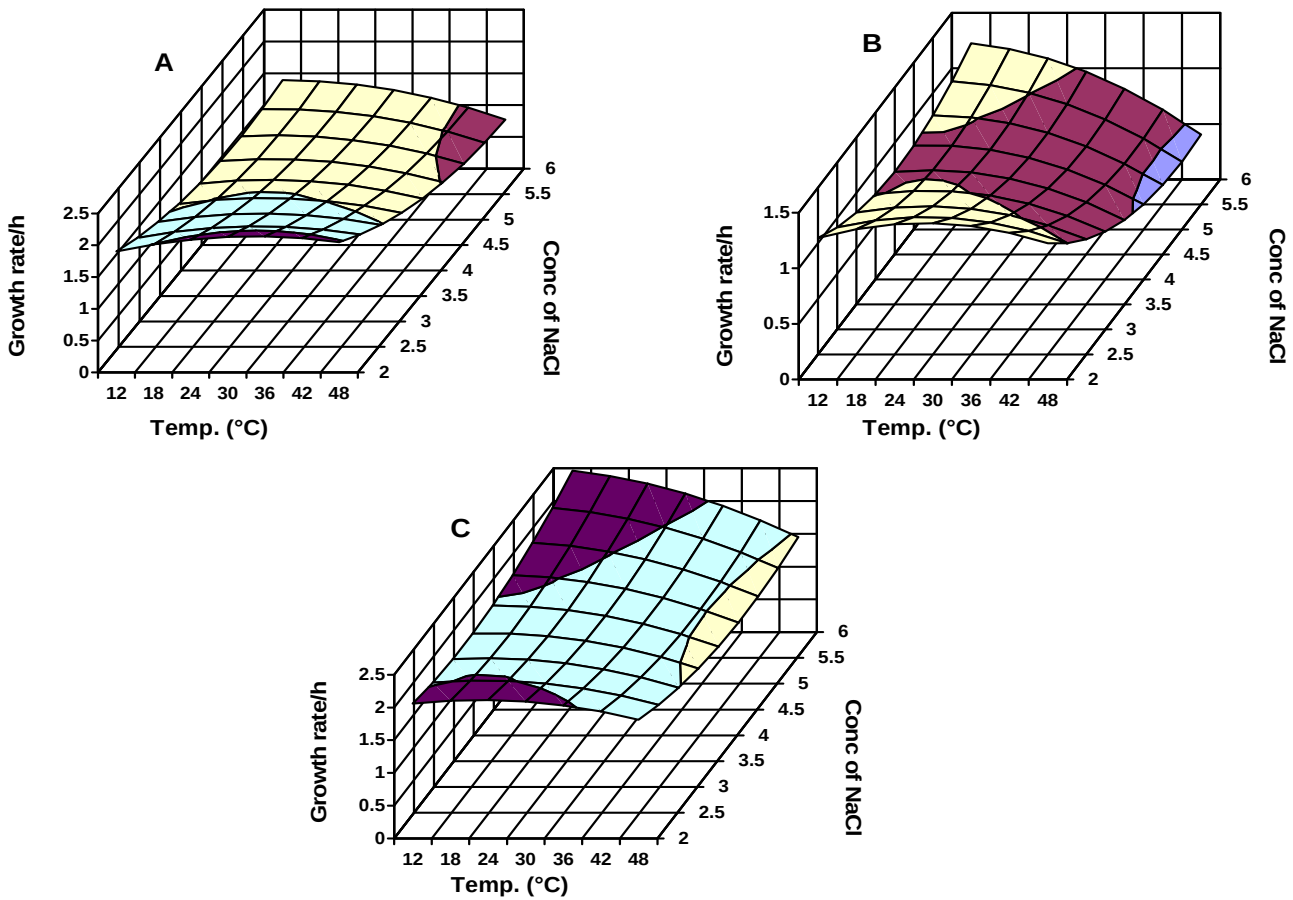


Figure 18 Response surface plots for GR of vegetative cells of *B. cereus* CFR 1534 grown in BHI broth at pH levels of 5.5 [**A**], 6.5 [**B**] and 7.5 [**C**] $\log_{10}$ CFU/ml, respectively

combinations of incubation temperature (12-48°) and NaCl concentration (2-6%), GR values were in the range of 1.1 to 2.1/h.

The GR values obtained over the range of influencing factors at pH 6.5 were lower than those obtained at pH 5.5. The GR of less than 1.0/h (to be more accurate less than 0.9/h) was variable which could be observed as follows (**Figure 18B**):

i) 48°C and NaCl levels of 3 to 6% (with the lowest at 6% NaCl)

ii) 42°C and NaCl levels of 3.5 to 6%

iii) 36°C and NaCl levels of 4 to 6%

iv) 30°C and NaCl levels of 4.5 to 6%

At all other combinations of incubation temperatures and NaCl concentrations, the GR values were in the range of 0.9 to 1.4/h.

At pH 7.5 (**Figure 18B**), the GR values generated for vegetative cells of *B. cereus* CFR 1534 over the range of combinations of variables by response surface were of higher values ranging from a minimum of 1.3/h at 48°C and NaCl levels of 4 to 5.5% and a maximum of 2.0 to 2.5/h at the combinations of (i) 12-36°C with NaCl levels of 2 and 6% and (ii) 18-24°C with NaCl levels of 2.5, 4.5, 5, 5.5 and 6%.

In the background of developing HACCP protocols in the food chain, the response surface plots do provide basic data like the one relating to LPD and GR of vegetative cells of a potent toxigenic native food isolate of *B. cereus* as in the present study. A detailed analysis of the plots could make it possible to render foods with extended shelf life, as it is LPD and GR of a pathogen which enables the organism to reach risk causing viable population levels.

4.3.3.3 Lag phase duration of spores of *B. cereus*

In contrast to the vegetative cells, the LPD of spores would include the time for germination and outgrowth of the spores to transform to vegetative cells prior to going in to proper growth phase. In a similar manner to the previously described LPD and GR of vegetative cells, even for the spores, the data obtained from the experimental trials was subjected to the model of Baranyi and Roberts (1994) and the derived experimental LPD values were subjected to multiple regression analysis. The derived predicted values are presented in **Table 21**. The experimental LPD values ranged from a lowest of 5.8 h at a combination of incubation temperature of 42°C, pH of 6.5 and NaCl level of 4% and to a highest of 20.5 h at 22°C, pH of 6.5 and 4% NaCl level. The derived regression coefficients, their standard errors and *P* values are detailed in **Table 22**. The R^2 value of 0.81 indicates a fairly high degree of correlation between observed and predicted values. The linear coefficients of temperature and cross product coefficient (temperature x temperature) were significant with very small *P* values ($P < 0.05$). The other remaining term coefficients were not significant. It could be visualized that incubation temperature appeared to be the primary influencing factor for the LPD of spores of *B*. *cereus* CFR 1534.

A few of the earlier research investigations have reported higher LPD values like 12.9 h at a combination of 45°C, pH of 6.5 and 4% NaCl level (Ng and Schaffner, 1997). A LPD of 16.85 h was reported in studies on the effect of environmental factors on the growth kinetics of spores of *B*. *cereus* ATCC 7004 at a temperature of 30°C, pH 6.0 and 3% NaCl (Martinez et al. 2007).

Table 21 Experimental and predicted LPD values of spores of *B. cereus* CFR 1534

Treatment number	Temperature (°C)	pH Level	NaCl conc. (%)	Experimental LPD (h) ± SD	Predicted LPD (h)
1	26.1	5.9	2.8	10.5 ± 1.6	12.4
2	37.9	5.9	2.8	7.1 ± 2.5	5.6
3	26.1	7.1	2.8	13.0 ± 1.4	13.8
4	37.9	7.1	2.8	8.9 ± 4.5	7.9
5	26.1	5.9	5.2	15.2 ± 3.8	16.1
6	37.9	5.9	5.2	12.7 ± 1.6	11.9
7	26.1	7.1	5.2	13.9 ± 0.1	15.5
8	37.9	7.1	5.2	14.3 ± 0.8	12.3
9	22.0	6.5	4.0	20.5 ± 1.0	17.4
10	42.0	6.5	4.0	5.8 ± 0.1	8.9
11	32.0	5.5	4.0	11.5 ± 4.7	11.2
12	32.0	7.5	4.0	12.3 ± 1.6	12.7
13	32.0	6.5	2.0	7.0 ± 0.7	6.9
14	32.0	6.5	6.0	13.5 ± 0.8	13.6
15	32.0	6.5	4.0	7.8 ± 1.6	9.2
16	32.0	6.5	4.0	9.5 ± 2.6	9.2
17	32.0	6.5	4.0	7.8 ± 1.6	9.2
18	32.0	6.5	4.0	9.8 ± 1.3	9.2
19	32.0	6.5	4.0	10.7 ± 2.8	9.2
20	32.0	6.5	4.0	10.5 ± 0.3	9.2
21	32.0	6.5	4.0	8.3 ± 1.6	9.2

Table 22 Coefficients and associated standard error derived by multivariate analysis for LPD of spores of *B. cereus* CFR 1534

Source	Coefficient	SE	*P*
Intercept	180.1	81.7	0.05
T	-3.8	1.6	0.04
P	-34.1	20.3	0.12
N	1.0	7.7	0.90
T^2	0.04	0.01	0.02
T x P	0.1	0.2	0.72
P^2	2.7	1.4	0.12
P x N	-0.7	1.0	0.49
N^2	0.3	0.4	0.47
N x T	0.1	0.1	0.36
R^2	0.81		

T, incubation temperature (°C); P, pH level; N, NaCl concentration; R^2, Coefficient of determination

As in the case of LPD of vegetative cells, the derived coefficients were utilized to generate response surface plots for spores of vegetative cells of *B. cereus* CFR 1534 at 3 defined pH levels of 5.5, 6.5 and 7.5. The proposed equation was to be:

$LPD = 180.138 – 3.81048T - 34.1302P + 0.987229N + 0.03964T^2 + 0.072564T*P + 2.714316P^2 - 0.68316P*N + 0.266079N^2 + 0.094191N*T$

where LPD is the lag phase duration; T is the incubation temperature; P is the pH level; and N is the sodium chloride concentration.

The individual response surface plots at the defined pH levels are shown in **Figure 19**.

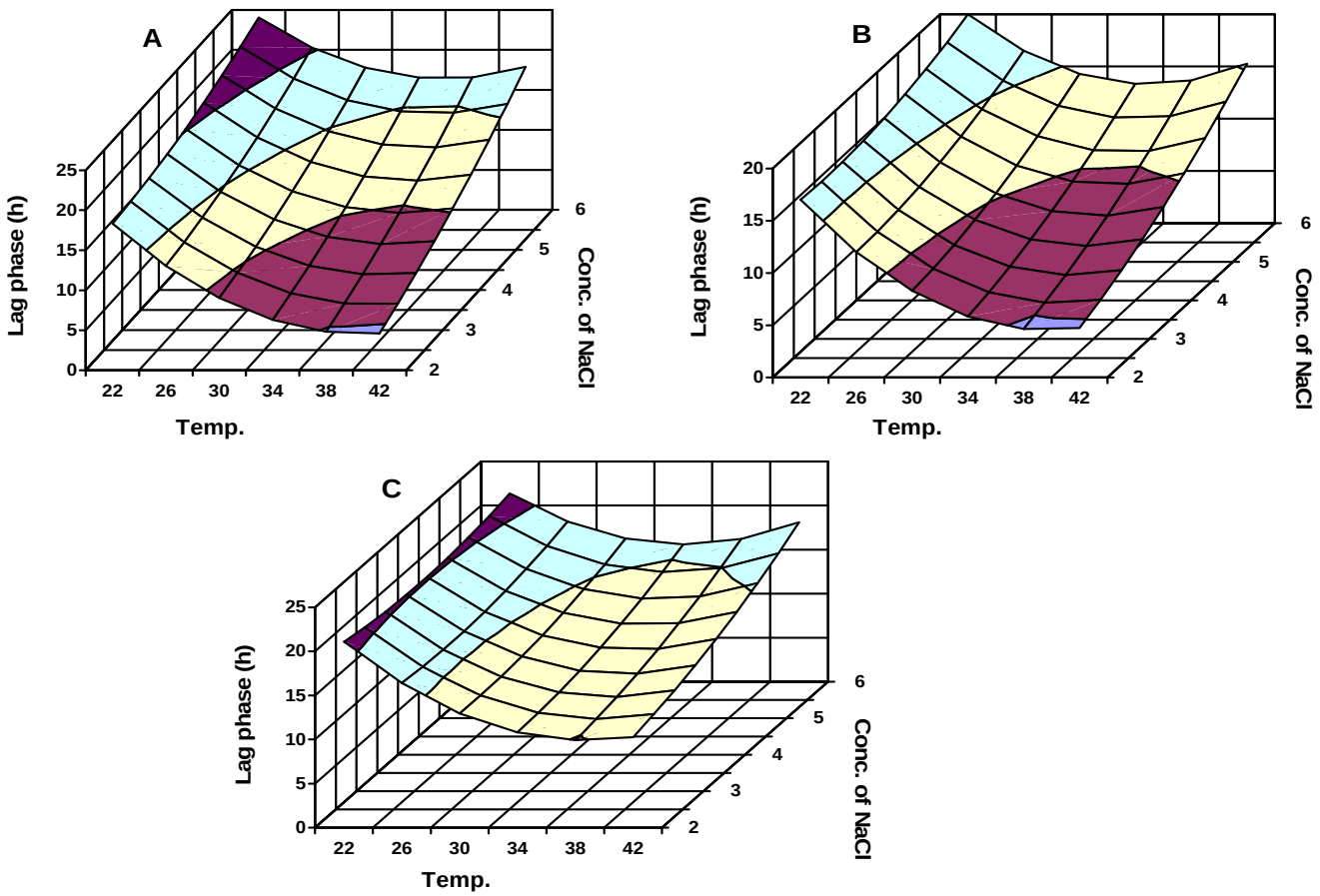


Figure 19 Response surface plots for LPD of spores of *B. cereus* CFR 1534 grown in BHI broth at pH levels of 5.5 [**A**], 6.5 [**B**] and 7.5 [**C**] $\log_{10}$ CFU/ml, respectively

In the case of spores of *B*. *cereus* CFR 1534 at pH levels of 5.5 and 6.5, the LPD values generated were in the range of 4.6 to 24.0 h. At pH 5.5, lower values (4.6 to 8.0 h) were recorded at incubation temperatures of 34-42°C and NaCl levels of 2, 2.5 and 3% (**Figure 19A)**. At all temperatures of the experimental design, the LPD values progressively increased with increasing levels of NaCl. At pH 6.5, lower LPD values of 4.6 to 8.0 h were observed at temperatures of 34-42°C and NaCl levels of 2, 2.5, 3 and 3.5% (**Figure 19B)**. The behaviour at other combinations was similar to that observed at pH 5.5, except for slight lower LPD values. Interestingly, at pH 7.5 (**Figure 19C)**, the LPD values ranged from a minimum of 9.9 to a maximum of 21.4 h, although this maximum value was lower than that observed (24.0 h) with pH 5.5 at a temperature of 22°C with 6% NaCl. At this pH of 7.5, at all the combinations of incubation temperatures and pH levels, the LPD values showed a progressive increase with increasing levels of NaCl.

Considering the nature of spores, definitely higher LPD values would be observed. However, with combination of the factors and variables used in the present experimental design, it would be possible to have protocols to extend the shelf life of food products.

4.3.3.4 Growth rate of spores of *B. cereus*

The experimental GR values for spores of *B*. *cereus* were derived through the use of model of Baranyi and Roberts (1994). The experimental GR values were subjected to multiple regression analysis and the derived predicted values are presented in **Table 23**. The experimental GR values ranged from 0.2 to 0.7/h. The lowest GR of 0.2 was observed at a combination of 22°C, pH 6.5 and 4% NaCl, whereas the highest GR of 0.7 was with a combination of 32°C, pH 6.5 and 4% NaCl.

Table 23 Experimental and predicted GR values of spores of *B. cereus* CFR 1534

Treatment number	Temperature (°C)	pH Level	NaCl conc. (%)	Experimental GR (value/h) ± SD	Predicted GR (value/h)
1	26.1	5.9	2.8	0.4 ± 0.5	0.5
2	37.9	5.9	2.8	0.5 ± 0.6	0.5
3	26.1	7.1	2.8	0.3 ± 0.9	0.2
4	37.9	7.1	2.8	0.4 ± 0.3	0.5
5	26.1	5.9	5.2	0.4 ± 0.5	0.4
6	37.9	5.9	5.2	0.3 ± 0.4	0.4
7	26.1	7.1	5.2	0.3 ± 0.3	0.3
8	37.9	7.1	5.2	0.6 ± 0.1	0.6
9	22.0	6.5	4.0	0.2 ± 0.1	0.3
10	42.0	6.5	4.0	0.6 ± 0.0	0.5
11	32.0	5.5	4.0	0.6 ± 0.1	0.5
12	32.0	7.5	4.0	0.3 ± 0.0	0.4
13	32.0	6.5	2.0	0.4 ± 0.1	0.4
14	32.0	6.5	6.0	0.4± 0.4	0.4
15	32.0	6.5	4.0	0.4 ± 0.2	0.5
16	32.0	6.5	4.0	0.6 ± 0.1	0.5
17	32.0	6.5	4.0	0.7 ± 0.1	0.5
18	32.0	6.5	4.0	0.5 ± 0.2	0.5
19	32.0	6.5	4.0	0.5 ± 0.2	0.5
20	32.0	6.5	4.0	0.5 ± 0.3	0.5
21	32.0	6.5	4.0	0.4 ± 0.2	0.5

The derived regression coefficients, their standard errors and *P* values are detailed in **Table 24**. The R^2 value of 0.58 indicates a poor correlation between the observed and predicted values. The coefficient values and cross product coefficients were found to be not significant. None of the factors studied appeared to have any major influence on the spores of *B*. *cereus* CFR 1534.

Table 24 Coefficients and associated standard error derived by multivariate analysis for GR of spores of *B*. *cereus* CFR 1534

Source	Coefficient	SE	*P*
Intercept	0.14	4.8	0.98
T	-0.03	0.1	0.80
P	0.35	1.2	0.78
N	-0.18	0.5	0.70
T^2	0.00	0.0	0.13
T x P	0.02	0.0	0.14
P^2	-0.09	0.1	0.30
P x N	0.06	0.1	0.34
N^2	-0.03	0.1	0.14
N x T	0.00	0.0	0.74
R^2	0.58		

T, incubation temperature (°C); P, pH level; N, NaCl concentration; R^2, Coefficient of determination

Ng and Schaffner (1997) in their studies reported a lower GR value (0.19/h) at a combination of 45°C, pH 6.5 and 1% NaCl. In a similar study (Martinez et al. 2007), a GR of 0.26/h was obtained at a temperature of 30°C, pH 6.0 and 3% NaCl.

The coefficients derived by multivariate analysis were utilized to generate response surface plots for GR of spores of *B. cereus* CFR 1534 as a function of varying incubation temperatures and NaCl concentrations at 3 defined pH levels of 5.5, 6.5 and 7.5. The response surface model for GR was proposed to be:

$GR = 0.136477 - 0.02781T + 0.347615P - 0.1774N - 0.00141T^2 + 0.018891T*P$

$- 0.09394P^2 + 0.05816P*N - 0.03411N^2 + 0.00203N*T$

where GR is the growth rate; T is the incubation temperature; P is the pH level; and N is the sodium chloride concentration.

The individual response surface plots at the defined pH levels are shown in **Figure 20**.

The surface plots generated for spores of *B. cereus* CFR 1534 did reflect the earlier observation with derived coefficients, wherein it was indicated that no factor could be a primary influencing factor. However, the GR values obtained were quite interesting. At pH 5.5, the GR values ranged from a minimum of 0.05/h at 42°C and 6% NaCl to a maximum of 0.52/h at 30°C and 3% NaCl. The trend in behaviour was an increase from the initial GR values at 2-3% NaCl levels and at incubation temperatures of 22-42°C, after which at levels of 3.5-6.0% NaCl, the GR values progressively decreased with the lowest value (at specific temperature) being with 6% NaCl (**Figure 20A**). In an almost similar manner, at pH 6.5, the lowest GR value of 0.07/h was at 22°C and 6% NaCl and the highest of 0.56/h was at 34°C with 4% NaCl and 38°C with 4.5% NaCl, respectively. In comparison to pH 5.5, the increase from the initial GR values at temperatures of 22 and 26°C was observed with NaCl levels from 2-3.5%, after which the values progressively decreased. At temperatures of 30-42°C, this increase was observed with NaCl levels of 2-4%, after which there was a decrease in GR values (**Figure 20A**)

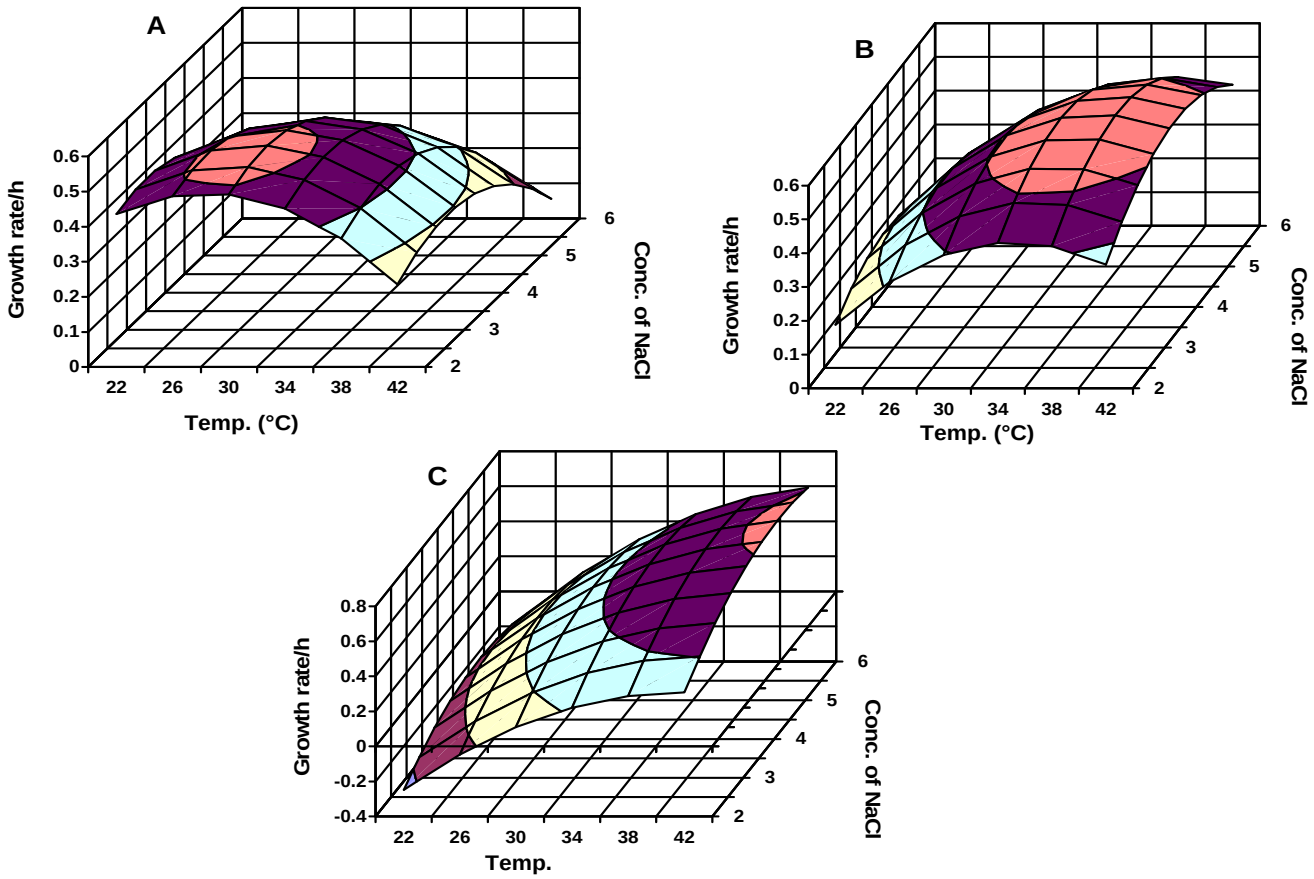


Figure 20 Response surface plots for GR of spores of *B. cereus* CFR 1534 grown in BHI broth at pH levels of 5.5 [**A**], 6.5 [**B**] and 7.5 [**C**] $\log_{10}$ CFU/ml, respectively

At pH 7.5, the GR values generated were negative at a temperature of 22°C with NaCl levels of 2-6% with the lowest of -0.04/h (4.5% NaCl). A similar trend to that of pH 5.5 and 6.5 with respect to initial increase and subsequent decrease does exist at the temperatures included in the design, except for 22°C. However, the increase in GR values extends from 2-5% NaCl levels and shows marginal decrease at 5.5 and 6.0% NaCl levels (**Figure 20C**).

The cultures of *B*. *cereus* which exist as vegetative cells and spores have better survival and growth ability in a diverse range of food matrices. The generated LPD and GR values did reveal the versatility of native food isolate *B*. *cereus* CFR 1534. The derived models can be used to predict responses within the range of influencing factors studied and for which experimental data were collected. A similar view point has been proposed much earlier in this regard (Buchanan et al. 1993). The model derived in the study showed a good predictive ability of the data obtained from the published literature. The model was found to be significant and the predicted values were found to be in good agreement for LPD and moderately fitting for GR values. The study underlines the synergistic interaction of the three factors (temperature, pH and salt concentration) on LPD and GR of the selected potent toxigenic native food isolate of *B*. *cereus* CFR 1534. The results obtained with broth system would enable to develop HACCP protocols in the food chain towards providing microbiologically safe foods to the human population.

4.4 BEHAVIOURAL PATTERN OF *BACILLUS CEREUS* IN SELECTED FOOD MATRICES

Foods have been an integral part of human life. India being a home of cultural diversity, the Indian palate is well acclimatized to a diverse range of foods, both traditional and non-traditional. Irrespective of this wide spectrum of foods, the primary focus in this complex food chain is to make available safe and healthy food with no associated risk causing factors. In this scenario, microbial food safety becomes a serious public health concern. It is very well known and also established that bacterial foodborne pathogens are the causative agents of health hazards through their survival, growth in foods and production of undesirable metabolites. In the background of profile of diverse range of foods, the behaviour of individual bacterial species in the prevailing complexity of specific food matrices would either make the foods risk-free or risk-associated.

The cultures of *B*. *cereus* are known to be prevalent under varied conditions encountered in the food chain. It is well documented that this bacterial species has a very close affinity with cereal and legume-based food commodities, followed by milk and milk products and other foods. Often, cereals and legumes are used as nutritious foods primarily as dehydrated premixes for further use based on consumption needs. Invariably, in the traditional methods of preparation (pre-processing) of such foods and subsequent storage and use (post-processing), there occurs ample opportunities for contamination with *B*. *cereus*. Being a dehydrated product, the focus would be on spores as they can survive under adverse conditions (low water activity) for longer storage periods. In a similar manner, many heat processed ready-to-use foods are also open to chance contamination with *B*. *cereus*, which may be due to faulty / under-processing or post-processing weak linkages in the food chain.

In the present experimental trials, an attempt has been made to assess the behavioural pattern of vegetative cells and spores of a native potent toxigenic isolate of *B*. *cereus* CFR 1534 as a function of temperature, initial inoculum and storage

period was studied using Fractional Factorial Design in two food systems namely (i) a traditional cereal legume based dehydrated nutritious food product based on millet and legume, locally known as *Ragi hurittu* and (ii) a ready-to-use chocolate milk, which is a cocoa-based beverage with reasonable total solids.

4.4.1 MATERIALS

All glasswares, media and other materials used in the present study were either wet sterilized or dry sterilized. Wet sterilization was carried out at 121°C for 20 min in an autoclave and dry sterilization at 180°C for 4 h in a Hot Air Oven. All bacteriological media used were those of dehydrated media procured from Hi-Media Lab., Mumbai, India (Hi-Media, 2003). The media were prepared as per manufacturer's instructions. The water used in the experimental trials was Milli-Q water (A10 Elix 3, Millipore Corporation, Billerica, USA).

4.4.1.1 Bacterial (test) culture

This included a native food isolate of *B*. *cereus* CFR 1534 (**Table 2 under 4.1.3.1; pp 99**). The culture was maintained at 6°C on brain heart infusion (BHI) agar slants and propagated twice successively in BHI broth at 37°C prior to use in experimental trials.

4.4.1.2 Diluent

The diluent used was 0.85% normal saline as described earlier under **4.1.1.3.1** (**pp 76**).

4.4.1.3 Selected foods

These included:

(i) *Ragi hurittu*: This is a traditional cereal legume based dehydrated food product, locally known as *Ragi hurittu* prepared from the mixed blend of roasted finger millet

(*Eleucine coracana*) and Bengal gram (*Cicer aerietinum*) in the ratio of 2:1. It is consumed in the form of porridge or dumpling with the addition of lukewarm milk and if required, sugar or jaggery. ***This product will be mentioned as Ragi hurittu in the present experimental study and elsewhere in this thesis***. The powdered *Ragi hurittu* is normally kept stored under ambient conditions in a cool and dry place, both at house-hold and market places. The final prepared form made as and when needed. It serves as nutritious and most sought after delicacy especially for infants, convalescents and geriatric population.

(ii) Chocolate milk: Chocolate milk is a sweetened cocoa-based milk beverage and is available commercially. It contains cocoa powder, a sweetener such as sugar, chocolate syrup, starch and salt. It is kept under refrigerated conditions and consumed either cold or warm.

For use in the present study, commercial brands of *Ragi hurittu* and chocolate milk were obtained from the local market. The use of above-mentioned two products in the present experimental trials has been described under specific sections of methodology.

4.4.1.4 Bacteriological media

4.4.1.4.1 Nutrient broth and agar

This was prepared as described earlier under **4.1.1.7.7** (**pp 80**) and used in this study.

4.4.1.4.2 Nutrient - egg yolk agar

To the requisite quantity of melted and cooled (tempered to 50°C) nutrient agar medium, 1% egg yolk emulsion (prepared under aseptic condition) was added, mixed well and poured into pre-sterilized petri plates. Poured plates were allowed to remain for 30 min at ambient temperature for solidification of medium. Poured plates were used in the experimental trials within 24 h of preparation.

4.4.1.5 PCR primers

This included the oligonucleotide primers (Pi-PLC) designed for Phosphatidylinositol phospholipase C (*pi-plc*) as described earlier under **4.1.1.9** (**pp 83**).

5′AGTATGGGGAATGAC3′ 342 bp amplicon

5′ACAATTTTCCCACGA3′

4.4.1.6 Molecular biology requisites

These were the same as described earlier under **4.1.1.8** (**pp 82 and 83**).

4.4.2 METHODOLOGY

4.4.2.1 Inoculum preparation of vegetative cells and spores of test culture

The requisite suspension of vegetative cells and spores was prepared as described earlier under **4.2.2.2** (**pp 135**) and **4.2.2.3** (**pp 136**), respectively.

4.4.2.2 Experimental design in selected foods

Ragi hurittu: The experimental design for **vegetative cells** was a complete 3 x 3 x 3 factorial of initial inoculum (3.3, 5.3 and 7.3 $\log_{10}$ CFU/g), storage temperature (20, 30 and 40ºC) and storage period (2, 4 and 6 d). For **spores**, the experimental design was a complete 3 x 3 x 5 factorial of initial inoculum (3.3, 5.3 and 7.3 $\log_{10}$ CFU/g), storage temperature (20, 30 and 40ºC) and storage period (2, 4, 6, 8 and 10 d).

Requisite quantity of *Ragi hurittu* was dispensed into glass beakers and sterilized by autoclaving at 121°C for 30 min and cooled to ambient temperature. This was then spiked and mixed thoroughly under aseptic conditions with requisite inoculum (vegetative cells or spores) of *B*. *cereus* CFR 1534 to give the desired level of initial inoculum as per the experimental design. The experimental blended product was dispensed in 50 g quantities in to requisite number of UV exposed 200 gauge PP pouches of 25 x 18 cm^2 dimension and heat sealed. Individual sets of the inoculated packs were stored at the respective pre-selected temperatures. The

samples were enumerated for the viable counts of the specific test culture at the end of specified storage periods.

Chocolate milk: The experimental design for vegetative cells and spores was a complete 3 x 3 x 3 factorial of initial inoculum (3.3, 5.3 and 7.3 $\log_{10}$ CFU/g), storage temperature (20, 30 and 40ºC) and storage period (2, 4 and 6 d).

Requisite quantity (100 ml) of commercial brand of chocolate milk was transferred into pre-sterilized conical flasks and steamed for 30 min and cooled to ambient temperature. This sample was spiked with vegetative cells or spores of *B. cereus* and stirred well under aseptic conditions to get uniform level of target initial inoculum. The experimental blended product was dispensed in 50 ml quantities in to requisite number of aluminum foil covered pre-sterilized 100 ml glass beakers. Individual sets of the inoculated packs were stored at the respective pre-selected temperatures. The samples were enumerated for the viable counts of the specific test culture at the end of specified storage periods.

4.4.2.3 Enumeration of viable counts of *B. cereus*

The respective set of experimental samples *Ragi hurittu* and chocolate milk (spiked with vegetative cells and spores) were enumerated for the viable counts of inoculated *B. cereus*. Aliquots of the samples (11 g for *Ragi Hurittu* and 11 ml for chocolate milk) were blended with 99 ml of 0.85% sterile saline to give an initial dilution of 1:10. Aliquots of 0.1 ml of the appropriate dilutions were surface plated on pre-poured plates of nutrient agar in duplicates. The inoculated plates were incubated at 37°C for 24-48 h. Characteristic colonies of *B. cereus* appearing in the incubated plates were counted and expressed as average $\log_{10}$ CFU/g. The experimental trials were carried out independently, in triplicate and the mean values with statistical analysis were considered in presenting the data.

4.4.2.4 Modelling the behaviour of *B. cereus* and statistical analysis

The experimental data relating to the viable counts of the test cultures of *B. cereus* obtained from the experimental design were subjected to multiple regression analysis and predictions of microbial behaviour was achieved by partial least square regression. All calculations were performed in Microsoft Excel Program (Version 8.0, 2003, Microsoft Corporation, Redmond, WA, USA). The derived equation for individual experimental products studied was as follows:

$CFU_{prd} = X_0 + X_1(IC) + X_2(T) + X_3(St) + X_4(IC^2) + X_5(IC \times T) + X_6(T)^2 + X_7(T \times St) + X_s(St)^2 + X_9(St \times IC)$

where X_0 is the intercept, X_1, $-X_9$ are the coefficients, IC is the initial inoculum level, T is the storage temperature (°C) and St is the storage period (d). The coefficients derived by mulitvariate analysis were used to generate response surface models, which provide predictions for the populations of the test organism over a range of variables within the experimental domain.

Statistical testing of the model was performed using analysis of variance (ANOVA), which was used to test the significance and adequacy of the model (Microsoft Excel Program, Version 8.0, 2003, Microsoft Corporation, Redmond, WA, USA). ANOVA resulted in coefficient of determination (R^2). The coefficient values derived by multivariate analysis were calculated and tested for significance. Those with very small *P* values ($P < 0.05$) were considered significant and others as not significant.

4.4.2.5 Detection of *B. cereus* in food matrices by PCR and lecithinase activity

The test culture of *B. cereus* CFR 1534 was pre-grown in requisite quantity of *Ragi hurittu* and chocolate milk to get appropriate population levels of the inoculated culture in 72 h at 30°C (the numbers were evaluated by viable plate count).

PCR detection: Pre-grown samples of both the products in 10 g/ml quantities were suspended in 10 ml of sterile water. From this, 1.5 ml aliquots were taken to extract DNA by the method of Schraft and Griffiths (1995) as described earlier under **4.1.2.3.1** (**pp 91 and 92**). Conventional uniplex PCR amplification with Pi-PLC primers and analysis of the amplified products were performed as described under **4.1.2.3.2 (pp 92)** and **4.1.2.3.5** (**pp 94 and 95**), respectively.

Assay for lecithinase: Experimental (pre-grown with test culture) samples of both the products in 10 g/ml quantities were suspended in 10 ml of sterile water and subjected to centrifugation at 10000 rpm for 10 min at 20°C. The resulting supernatant in 100 µl aliquots were added into individual wells formed on pre-poured nutrient – egg yolk agar plates. The plates were incubated for 24-48 h at 37°C and observed for zone of egg yolk precipitation (halo formation) surrounding the individual wells as a positive reaction for lecithinase activity elaborated by *B*. *cereus* in the food product.

4.4.3 RESULTS AND DISCUSSION

4.4.3.1 Behaviour of vegetative cells of *B*. *cereus* in *Ragi hurittu*

The viable counts of *B*. *cereus* CFR 1534 introduced as vegetative cells in a traditional product – *Ragi hurittu* as per the outlined experimental design were recorded and the average counts were subjected to multiple regression analysis to derive the model fits to predict the behaviour of this bacterial isolate. The experimental and predicted counts are shown in **Table 25**. The derived regression coefficients, their standard errors and *P* values are detailed in **Table 26**. The R^2 value of 0.86 indicates a fairly high degree of correlation between experimental and predicted values. On the basis of values obtained for the linear and/or cross-product coefficients, it would appear that initial numbers of *B*. *cereus* vegetative cells introduced into the product would be the primary potential influencing factor under

the conditions of storage temperature and period to cause any risks to be associated with a product known to harbor vegetative cells of *B*. *cereus*.

The experimental data revealed that at storage temperatures of 20 and 30°C and initial inoculum of 3.3 $\log_{10}$ CFU/g, there was an increase of almost 2.0 logs in the viable count of *B*. *cereus* in the initial storage period of 2 d, after which the numbers remained same even till 6 d of storage. With initial levels of 5.3 and 7.3 $\log_{10}$ CFU/g, there was no appreciable increase visualized during storage period. Similarly, at storage temperature of 40°C, there was no appreciable change in the viable counts from the initial levels introduced in to the product. An observation of interest was that with initial inoculum level of 7.3 $\log_{10}$ CFU/g and at 3 storage temperatures, there was a marginal decrease in the viable count and at 6^{th} day of storage this decrease was quite appreciable at 40°C (**Table 25**).

The coefficients derived by multivariate analysis were used to generate response surface plots for the behaviour of vegetative cells of *B*. *cereus* CFR 1534 as a function of varying storage periods and temperatures at 2 defined initial levels of 4.3 and 6.3 $\log_{10}$ CFU/g. The response surface model for the viable counts was proposed to be:

$LVC = 5.103 - 0.122T + 0.899IC - 0.066t + 0.001T^2 + 0.006T*IC - 0.054IC^2 - 0.013IC*t - -0.022t^2 - 0.001t*T$

where LVC is the log of viable count; T is the storage temperature; IC is the initial inoculum level; and t is the storage period.

The individual response surface plots at the defined inoculums levels are shown in **Figure 21**.

In 2 d of storage at the selected storage temperatures for RSM, the viable count of *B*. *cereus* revealed a marginal increase of 1-2 logs from the initial level of 4.3 $\log_{10}$ CFU/g. Subsequently, the counts had a slow paced decreasing pattern during 3-6 days of storage (**Figure 21A**). A similar trend was also observed with the inoculum level of 6.3 $\log_{10}$ CFU/g, wherein the initial increase in viable count was less than 1.0 log in 2 d (**Figure 21B**).

Table 25 Experimental and predicted viable counts of *B. cereus* CFR 1534 spiked as vegetative cells in *Ragi hurittu*

Storage Temp. (°C)	Initial inoculum (log_{10} CFU/g)	Storage. period (d)	Exptl. viable count ± SD (log_{10} CFU/g)	Predicted viable count ± SD (log_{10} CFU/g)
20	3.3	2	5.7 ± 0.1	5.8
20	3.3	4	5.4 ± 0.1	5.6
20	3.3	6	5.3 ± 0.1	5.2
20	5.3	2	7.2 ± 0.2	6.9
20	5.3	4	6.8 ± 0.1	6.6
20	5.3	6	6.1 ± 0.2	6.1
20	7.3	2	7.3 ± 0.1	7.5
20	7.3	4	7.1 ± 0.1	7.2
20	7.3	6	6.6 ± 0.2	6.6
30	3.3	2	5.7 ± 0.1	5.4
30	3.3	4	5.4 ± 0.2	5.1
30	3.3	6	4.3 ± 0.1	4.7
30	5.3	2	6.3 ± 0.0	6.6
30	5.3	4	5.8 ± 0.1	6.3
30	5.3	6	5.8 ± 0.2	5.8
30	7.3	2	7.2 ± 0.1	7.3
30	7.3	4	7.2 ± 0.1	6.9
30	7.3	6	6.8 ± 0.1	6.4
40	3.3	2	4.9 ± 0.1	5.2
40	3.3	4	4.8 ± 0.1	4.9
40	3.3	6	4.7 ± 0.1	4.5
40	5.3	2	6.9 ± 0.0	6.5
40	5.3	4	5.4 ± 0.2	6.1
40	5.3	6	6.1 ± 0.4	5.7
40	7.3	2	7.2 ± 0.2	7.3
40	7.3	4	7.5 ± 0.0	7.0
40	7.3	6	5.8 ± 0.7	6.4

Table 26 Coefficients and associated standard error derived by multivariate analysis for vegetative cells of *B. cereus* CFR 1534 spiked in *Ragi hurittu*

Source	Coefficient	SE	*P*
Intercept	5.103	2.327	0.042
T	-0.122	0.109	0.277
IC	0.899	0.497	0.088
t	0.066	0.415	0.875
T^2	0.001	0.002	0.497
T x IC	0.006	0.006	0.323
IC^2	-0.054	0.042	0.211
IC x t	-0.013	0.030	0.674
t^2	-0.022	0.042	0.610
t x T	-0.001	0.006	0.922
R^2 0.86			

T, Storage temperature (°C); IC, Initial inoculum (log_{10} CFU/g); t, Storage period (d); R^2, Coefficient of determination

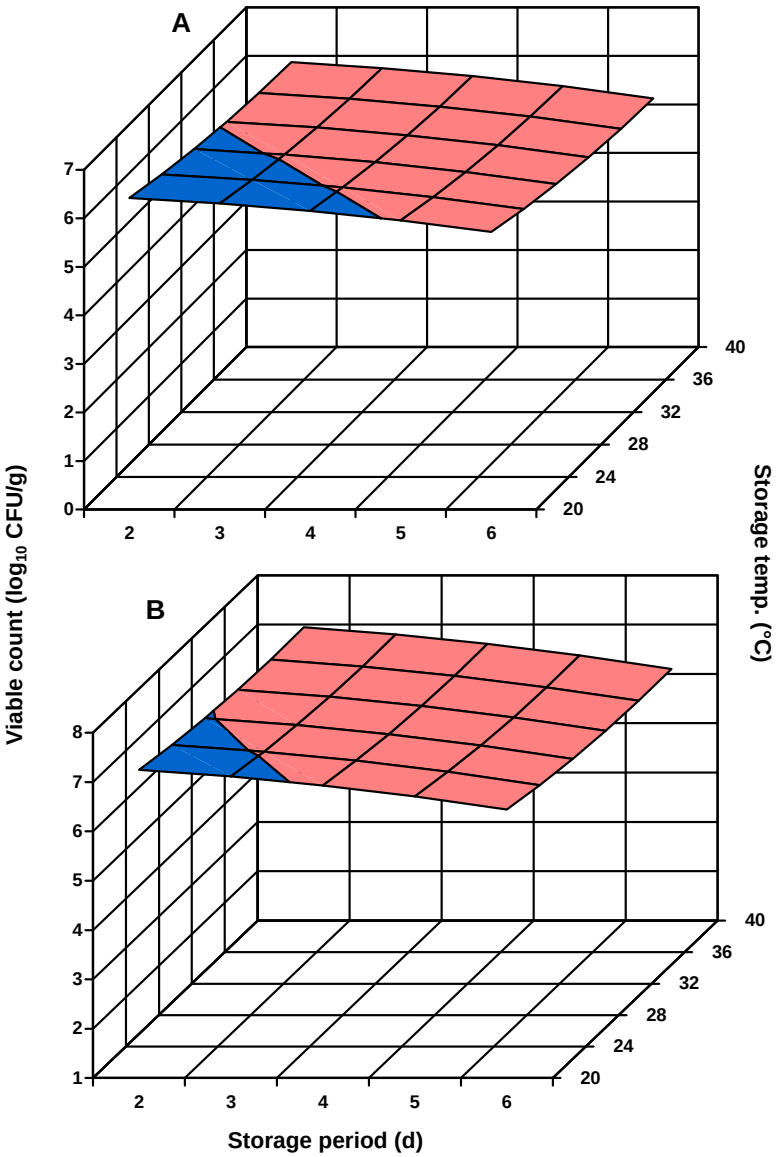


Figure 21 Response surface plots for the behaviour of vegetative cells of *B. cereus* CFR 1534 spiked in *Ragi hurittu* with initial inoculums levels of 4.3 [**A**] and 6.3 [**B**] $\log_{10}$ CFU/g, respectively

In the scenario of levels of contamination that tend to occur under normal commercial conditions in a food chain and the findings observed in this study, it could well be proposed that survival and growth of the bacterial species is very much dependent on the inherent ability of the organism to express normal growth phases under a diversified and complex environment like the food matrix. The basic phenomenon of a bacteria species is to enter a log phase from a minimum cell number, followed by stationary and death phases. This was very much evident in the response surface plots, wherein the initial increase occurred in the first 2 d of storage, after which the pattern was in accordance with the growth cycle. The initial increase in count was not appreciable in those samples with higher initial inoculums levels, since the population of bacterial species was almost near to those levels which the organism tends to achieve during log phase.

4.4.3.2 Behaviour of spores of *B. cereus* in *Ragi hurittu*

Considering that spores tend to remain for a longer period, in the present experimental trials, storage period included was 2-10 d (5 levels) and that of initial inoculum and storage temperature being of 3 levels. As in the case of vegetative cells, the average viable counts were subjected to multiple regression analysis. The experimental and resultant predicted counts (log_{10} CFU/g) are shown in **Table 27**. The derived regression coefficients, their standard errors and *P* values are presented in **Table 28**. The R^2 value of 0.97 indicates a high degree of correlation between experimental and predicted values. The linear coefficients (temperature and initial inoculum) and cross-product coefficients (T^2 and IC*t) were significant with very small *P* values ($P < 0.05$) and the other term coefficients were not statistically significant. However, it would appear that initial numbers of *B. cereus* spores introduced into the product would be the primary influencing factor.

Table 27 Experimental and predicted viable counts of *B*. *cereus* CFR 1534 spiked as spores in *Ragi hurittu*

Storage Temp. (°C)	Initial inoculum (log_{10} CFU/g)	Storage. period (d)	Exptl. viable count ± SD (log_{10} CFU/g)	Predicted viable count ± SD (log_{10} CFU/g)
20	3.3	2	3.3 ± 0.0	3.2
20	3.3	4	3.1 ± 0.0	3.2
20	3.3	6	2.9 ± 0.1	3.2
20	3.3	8	3.2 ± 0.1	3.2
20	3.3	10	3.4 ± 0.0	3.3
20	5.3	2	4.5 ± 0.0	4.3
20	5.3	4	4.7 ± 0.2	4.2
20	5.3	6	4.4 ± 0.1	4.1
20	5.3	8	3.9 ± 0.1	4.0
20	5.3	10	3.6 ± 0.3	3.9
20	7.3	2	6.3 ± 0.1	6.8
20	7.3	4	6.5 ± 0.0	6.6
20	7.3	6	6.3 ± 0.1	6.3
20	7.3	8	5.9 ± 0.3	6.1
20	7.3	10	6.0 ± 0.1	5.8
30	3.3	2	3.5 ± 0.1	3.5
30	3.3	4	3.4 ± 0.1	3.4
30	3.3	6	3.1 ± 0.2	3.4
30	3.3	8	3.6 ± 0.0	3.4
30	3.3	10	3.1 ± 0.1	3.4
30	5.3	2	4.2 ± 0.1	4.6
30	5.3	4	4.5 ± 0.1	4.4
30	5.3	6	3.9 ± 0.1	4.3
30	5.3	8	4.2 ± 0.1	4.1
30	5.3	10	4.1 ± 0.1	4.0
30	7.3	2	7.5 ± 0.0	7.1
30	7.3	4	7.0 ± 0.0	6.8
30	7.3	6	6.7 ± 0.0	6.5
30	7.3	8	6.3 ± 0.2	6.2
30	7.3	10	6.0 ± 0.2	6.0
40	3.3	2	3.5 ± 0.0	3.3
40	3.3	4	3.1 ± 0.3	3.3
40	3.3	6	3.5 ± 0.0	3.2
40	3.3	8	3.1 ± 0.2	3.2
40	3.3	10	3.3 ± 0.1	3.1
40	5.3	2	4.4 ± 0.0	4.5
40	5.3	4	4.4 ± 0.0	4.3
40	5.3	6	3.9 ± 0.1	4.1
40	5.3	8	3.7 ± 0.3	4.0
40	5.3	10	4.1 ± 0.1	3.8
40	7.3	2	6.9 ± 0.0	7.0
40	7.3	4	6.8 ± 0.0	6.7
40	7.3	6	6.5 ± 0.1	6.4
40	7.3	8	6.0 ± 0.1	6.1
40	7.3	10	5.6 ± 0.1	5.8

Table 28 Coefficients and associated standard error derived by multivariate analysis for spores of *B. cereus* CFR 1534 spiked in *Ragi hurittu*

Source	Coefficient	SE	*P*
Intercept	2.438	1.039	0.0257
T	0.116	0.050	0.027
IC	-0.806	0.230	0.001
t	0.127	0.093	0.179
T^2	-0.002	0.001	0.029
T x IC	0.000	0.003	0.895
IC^2	0.167	0.019	4.53E-10
IC x t	-0.032	0.008	0.000
t^2	0.001	0.005	0.792
t x T	-0.001	0.001	0.334
R^2 0.97			

T, Storage temperature (°C); IC, Initial inoculum (log_{10} CFU/g); t, Storage period (d); R^2, Coefficient of determination

Response surface plots were generated from the coefficients derived by multivariate analysis for the behaviour of spores of *B. cereus* CFR 1534 as a function of factors and variables. The response surface model for the viable counts was proposed to be and the generated plots are presented in **Figure 22**.

$LVC = 2.438 + 0.116T - 0.806IC + 0.127t - 0.002T^2 + 0.000T*IC + 0.167IC^2 - 0.032IC*t + 0.001t^2 - 0.001t*T$

where LVC is the log of viable count; T is the storage temperature; IC is the initial inoculum level; and t is the storage period.

The pattern of viable counts observed with the defined parameters of experimental factors and variables indicate that all the spores introduced in to the product would not be able to germinate, outgrow and become vegetative cells.

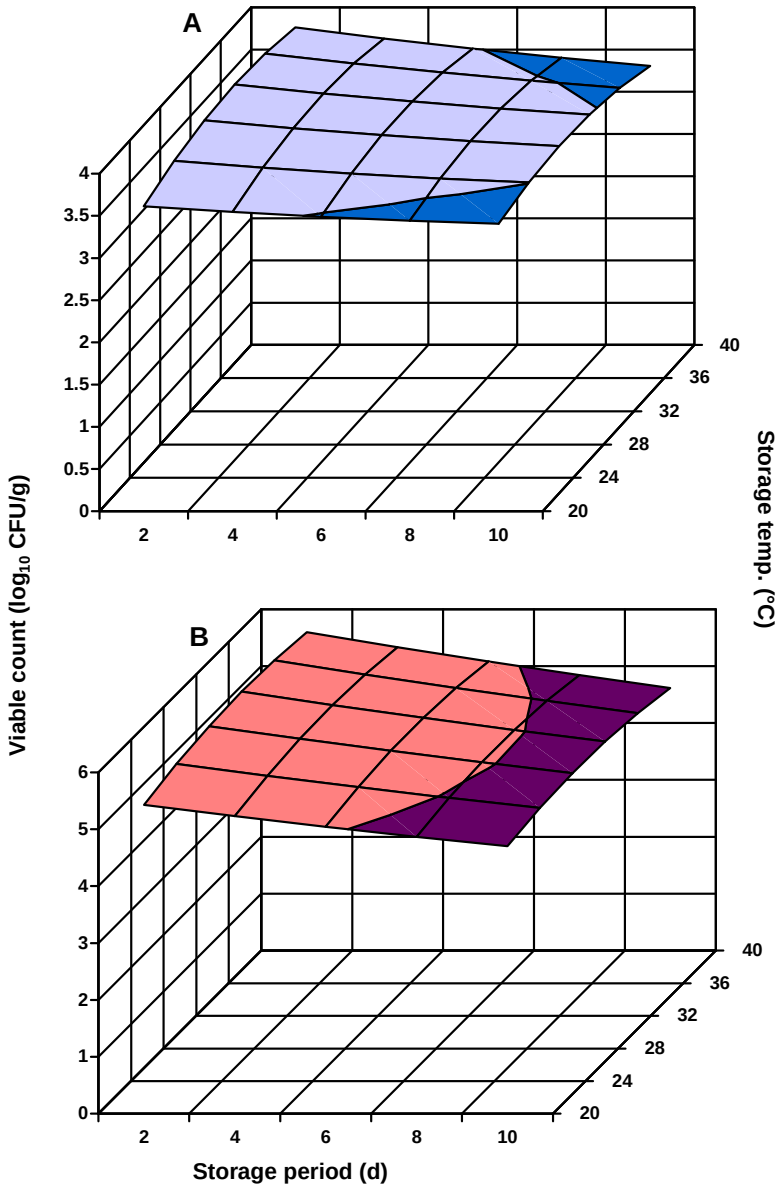


Figure 22 Response surface plots for the behaviour of spores of *B. cereus* CFR 1534 spiked in *Ragi hurittu* with initial inoculums levels of 4.3 [**A**] and 6.3 [**B**] log_{10} CFU/g, respectively

This may be due to the profile of product under study, wherein unfavourable parameters like low water activity may dominate over that of favourable attributes that could enable germination of spores. Irrespective of the initial inoculum levels (4.3 and 6.3 $\log_{10}$ CFU/g) of spores, at the selected storage temperatures, the trend was of a decreasing pattern right from 2 d of storage (**Figure 22 A and B**), wherein there was at least 1 log reduction in counts in a storage period of 10 d.

In general, dehydrated food products such as cereals and legumes dry premixes, like the one (*Ragi hurittu*) included in this study are regarded as microbiologically safe due to their low water activity. Although, these dehydrated products do not favour bacterial spore germination and growth of vegetative cells, the organisms tend to remain in a transient state for considerable period of time, which is more so with spores of *B*. *cereus*. Besides, the processing parameters of preparation of *Ragi hurittu* provide ample scope for contamination with *B*. *cereus* during milling of finger millet, blending with Bengal gram flour, exposure to conventional drying, moisture absorption during storage (due to simple and affordable packages) and hygienic practices during varied consumption pattern.

A few of the earlier research investigations have indicated the prevalence of *B*. *cereus* in flour and flour-based foods. Yusuf et al. (1992) observed the occurrence of enterotoxigenic *B*. *cereus* in Nigerian flour-based foods. Similarly, the occurrence has also been documented with wheat-based flour and dough (Rogers, 1978; Berghofer et al. 2003). It was of interest to know that survival and growth of *B*. *cereus* was the resultant of contamination during solar drying of finger millets (Kimanya et al. 2003). In the product of present study, the flour prepared out of atmospheric dried malted finger millet is the major ingredient. A minimum water activity of 0.98 to 0.95 is considered essential for growth and toxin production by *B*. *cereus*. However, addition of sugars in varied preparations of the basal product could lower the water activity. This condition may be slightly unfavourable for germination and outgrowth of spores and there are likely chances for recording low viable populations of *B*. *cereus*.

4.4.3.3 Behaviour of vegetative cells of *B. cereus* in chocolate milk

As against a traditional dry product of low water activity, in another set of experimental trials, the behaviour of vegetative cells of the same potent toxigenic native isolate of *B. cereus* CFR 1534 was studied in chocolate milk, which was slight viscous in nature. The average experimental viable counts subjected to multiple regression analysis resulted in predicted counts (**Table 29**). At the same time, regression coefficients, their standard errors and *P* values are presented in **Table 30**. The R^2 value of 0.99 indicates a very high degree of correlation between experimental and predicted counts. The linear and cross-product coefficients of initial inoculum (IC and IC^2) were significant with very small *P* values ($P < 0.05$) and the other term coefficients were not statistically significant. In this case, the initial numbers of *B. cereus* vegetative cells introduced into the product was the primary factor, which would cause the product to be marked under risk-associated.

Response surface plots were generated from the coefficients derived by multivariate analysis for the behaviour of vegetative cells of *B. cereus* under the defined influencing factors. The response surface model for the viable counts was proposed to be as follows and the generated plots are presented in **Figure 23**.

$$LVC = 2.010 - 0.060T + 0.520IC + 0.177t + 0.001T^2 + 0.001T*IC + 0.040IC^2 + 0.013IC*t - 0.012t^2 - 0.000t*T$$

where LVC is the log of viable count; T is the storage temperature; IC is the initial inoculum level; and t is the storage period.

The behavioural pattern of vegetative cells of *B. cereus* in chocolate milk was quite different as compared to *Ragi hurittu*. Irrespective of the level of initial inoculum introduced in to the product and also the storage temperatures, the viable counts revealed a slow paced increasing trend during storage period of 2-6 d with an almost 1 log increase achieved in 6 d (**Figure 23 A and B**).

Table 29 Experimental and predicted viable counts of *B*. *cereus* CFR 1534 spiked as vegetative cells in chocolate milk

Storage Temp. (°C)	Initial inoculum (log_{10} CFU/ml)	Storage. period (d)	Exptl. viable count ± SD (log_{10} CFU/ml)	Predicted viable count ± SD (log_{10} CFU/ml)
20	3.3	2	3.7 ± 0.6	3.7
20	3.3	4	3.8 ± 0.5	4.1
20	3.3	6	4.2 ± 0.3	4.3
20	5.3	2	5.8 ± 0.3	5.6
20	5.3	4	6.1 ± 0.3	6.0
20	5.3	6	6.1 ± 0.2	6.2
20	7.3	2	7.6 ± 0.3	7.7
20	7.3	4	8.2 ± 0.1	8.2
20	7.3	6	8.5 ± 0.3	8.5
30	3.3	2	3.7 ± 0.0	3.6
30	3.3	4	4.2 ± 0.4	3.9
30	3.3	6	4.2 ± 0.2	4.1
30	5.3	2	5.3 ± 0.3	5.5
30	5.3	4	5.6 ± 0.1	5.8
30	5.3	6	6.0 ± 0.1	6.1
30	7.3	2	7.6 ± 1.0	7.7
30	7.3	4	8.1 ± 0.6	8.1
30	7.3	6	8.4 ± 0.4	8.4
40	3.3	2	3.5 ± 1.2	3.7
40	3.3	4	4.0 ± 0.4	3.9
40	3.3	6	4.0 ± 0.3	4.1
40	5.3	2	5.6 ± 1.0	5.6
40	5.3	4	5.9 ± 0.1	5.9
40	5.3	6	6.1 ± 0.3	6.1
40	7.3	2	7.9 ± 0.1	7.8
40	7.3	4	7.9 ± 0.1	8.2
40	7.3	6	8.5 ± 0.3	8.5

Table 30 Coefficients and associated standard error derived by multivariate analysis for vegetative cells of *B*. *cereus* CFR 1534 spiked in chocolate milk

Source	Coefficient	SE	*P*
Intercept	2.010	0.949	0.049
T	-0.060	0.044	0.190
IC	0.520	0.202	0.019
t	0.177	0.169	0.308
T^2	0.001	0.001	0.226
T x IC	0.001	0.002	0.613
IC^2	0.040	0.017	0.031
IC x t	0.013	0.012	0.279
t^2	-0.012	0.017	0.500
t x T	-0.000	0.002	0.932
R^2 0.99			

T, Storage temperature (°C); IC, Initial inoculum (log_{10} CFU/ml); t, Storage period (d); R^2, Coefficient of determination

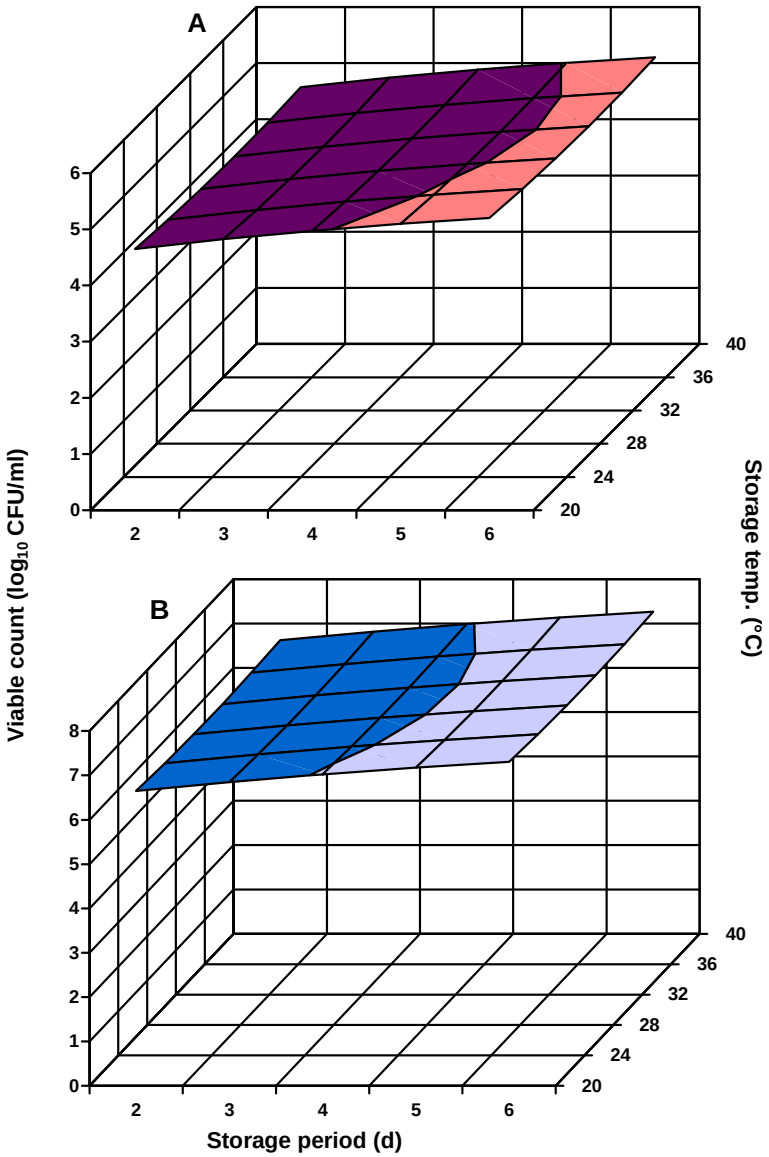


Figure 23 Response surface plots for the behaviour of vegetative cells of *B. cereus* CFR 1534 spiked in chocolate milk with initial inoculums levels of 4.3 [**A**] and 6.3 [**B**] $\log_{10}$ CFU/ml, respectively

4.4.3.4 Behaviour of spores of *B. cereus* in chocolate milk

The average viable counts obtained form the experimental trials were subjected to multiple regression analysis to derive the predicted model fit. The experimental and predicted viable counts for the spores of *B*. *cereus* CFR 1534 are detailed in **Table 31**. At the same time, values of regression coefficients, their standard errors and *P* values are presented in **Table 32**. The R^2 value of 0.99 gives an indication of a high degree of correlation between experimental and predicted counts. Only the linear coefficient of initial inoculum (IC) was significant with very small *P* values ($P < 0.05$) and the other term coefficients were not statistically significant. Accordingly, it appears that initial inoculum was the primary influencing factor to affect the behaviour of spores of *B*. *cereus* in the given food matrix.

The derived regression coefficients were used to generate data for developing response surface plots, as a means to describe the behavioural pattern of spores of *B*. *cereus* in chocolate milk. The response surface model for the viable counts was proposed to be as follows:

$$LVC = 1.791 - 0.021T + 0.582IC - 0.074t - 0.000T^2 + 0.004T*IC + 0.024IC^2 + 0.019IC*t + 0.007t^2 + 0.002t*T$$

where LVC is the log of viable count; T is the storage temperature; IC is the initial inoculum level; and t is the storage period.

The behavioural pattern that could be visualized was similar to that of vegetative cells, except for a slow paced increase during the storage period from 2-6 d at the defined storage temperatures. The initial viable counts reached in 2 d storage at temperatures of 20 to 40°C revealed a decreasing pattern, wherein the difference was highly marginal, after which the same paced increase in counts was visualized during 3-6 d of storage (**Figure 24 A and B**). The increase in counts was almost nearing to 1 log in 6 d of storage. The slow increase observed with spores is mostly related to the time taken by spores to germinate, outgrow and multiply as vegetative cells under the given environmental conditions.

Table 31 Experimental and predicted viable counts of *B*. *cereus* CFR 1534 spiked as spores in chocolate milk

Storage Temp. (°C)	Initial inoculum (log_{10} CFU/ml)	Storage. period (d)	Exptl. viable count ± SD (log_{10} CFU/ml)	Predicted viable count ± SD (log_{10} CFU/ml)
20	3.3	2	3.8 ± 0.3	3.8
20	3.3	4	3.7 ± 0.4	4.0
20	3.3	6	4.1 ± 0.0	4.2
20	5.3	2	5.6 ± 0.0	5.6
20	5.3	4	5.9 ± 0.1	5.8
20	5.3	6	6.3 ± 0.1	6.1
20	7.3	2	7.6 ± 0.1	7.6
20	7.3	4	7.9 ± 0.0	7.9
20	7.3	6	8.3 ± 0.2	8.3
30	3.3	2	3.8 ± 0.1	3.6
30	3.3	4	3.9 ± 0.3	3.8
30	3.3	6	4.1 ± 0.2	4.1
30	5.3	2	5.5 ± 0.3	5.5
30	5.3	4	5.7 ± 0.3	5.8
30	5.3	6	6.1 ± 0.1	6.1
30	7.3	2	7.5 ± 0.0	7.6
30	7.3	4	7.8 ± 0.2	7.9
30	7.3	6	8.3 ± 0.3	8.4
40	3.3	2	3.1 ± 0.1	3.4
40	3.3	4	3.7 ± 0.3	3.6
40	3.3	6	4.0 ± 0.1	3.9
40	5.3	2	5.2 ± 0.2	5.3
40	5.3	4	5.7 ± 0.2	5.7
40	5.3	6	5.9 ± 0.1	6.1
40	7.3	2	7.5 ± 0.5	7.5
40	7.3	4	8.1 ± 0.3	7.9
40	7.3	6	8.3 ± 0.2	8.4

Table 32 Coefficients and associated standard error derived by multivariate analysis for spores of *B*. *cereus* CFR 1534 spiked in chocolate milk

Source	Coefficient	SE	*P*
Intercept	1.791	0.803	0.039
T	-0.021	0.037	0.579
IC	0.582	0.171	0.003
t	-0.074	0.143	0.610
T^2	-0.000	0.000	0.605
T x IC	0.004	0.002	0.070
IC^2	0.024	0.014	0.122
IC x t	0.019	0.010	0.070
t^2	0.007	0.014	0.640
t x T	0.002	0.002	0.240
	R^2	0.99	

T, Storage temperature (°C); IC, Initial inoculum (log_{10} CFU/ml); t, Storage period (d); R^2, Coefficient of determination

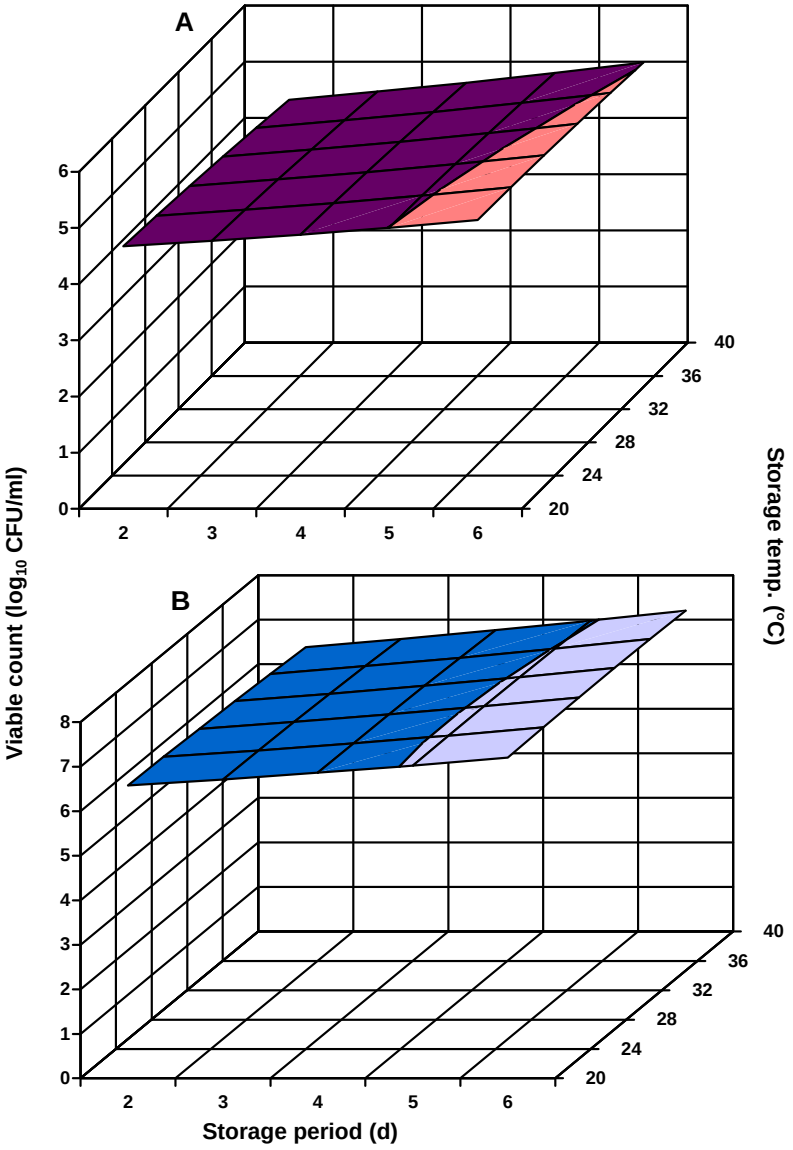


Figure 24 Response surface plots for the behaviour of spores of *B. cereus* CFR 1534 spiked in chocolate milk with initial inoculums levels of 4.3 [**A**] and 6.3 [**B**] $\log_{10}$ CFU/ml, respectively

The experimental trials included the detection of *pi-plc* gene of *B. cereus* by PCR through the use of Pi-PLC primers using experimental samples of *Ragi hurittu* and chocolate milk. Simultaneously, the pre-grown samples were also assayed for lecithinase activity. Both these experiments were undertaken so as to have a

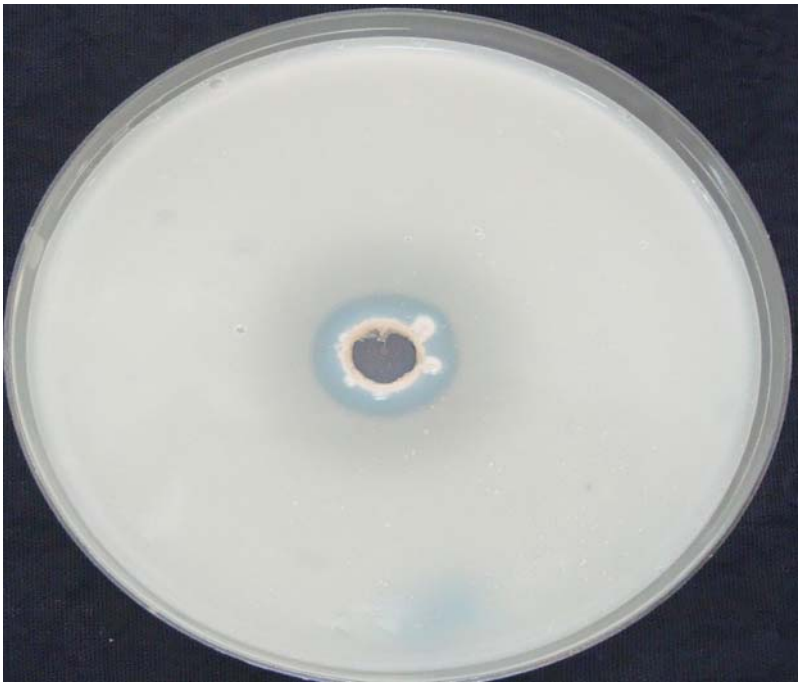
probable indication of toxigenic traits that could be associated with the growth of *B*. *cereus* in the products, which otherwise may appear to be sensorily acceptable. Both the experimental samples were negative for any amplification of Pi-PLC primers in PCR detection. However, the experimental samples of *Ragi hurittu* and chocolate milk revealed positive lecithinase activity in the assay plates (**Figure 25 A and B**).

Often, commercially available ready-to-consume milk-based products including chocolate milk, the one included in this study are open to contamination, particularly with vegetative cells and spores of *B*. *cereus*. Under conditions of temperature abuse during commercial practices of transit and storage as well as the specific consumption pattern, both the vegetative cells and spores of *B*. *cereus* can result in increased levels of viable population and a unsafe product for human consumption.

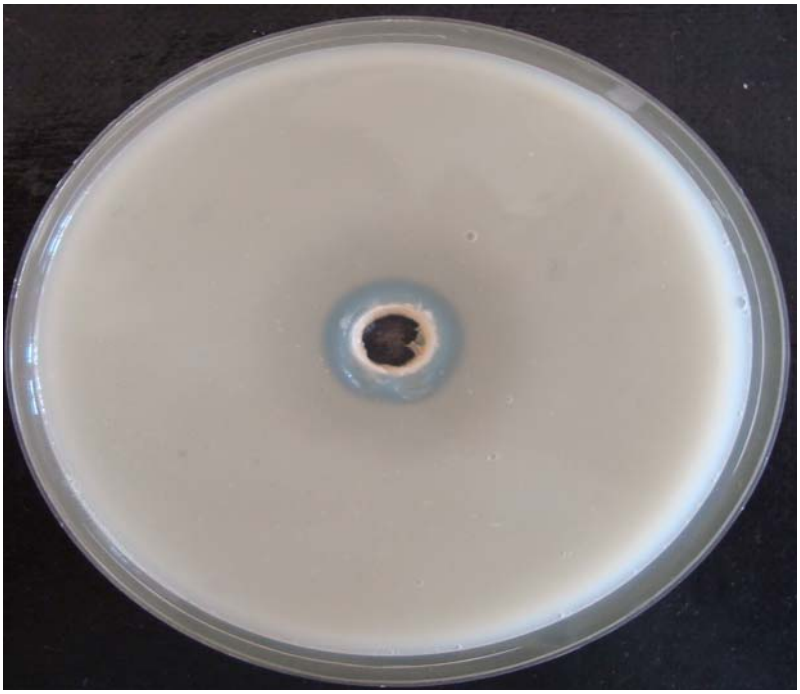
In one of the earlier studies by Feijoo et al. (1997), it was shown that there was a rapid growth of bacterial vegetative cells in whipping cream at a storage temperature of 32°C. Predictions using linear regression revealed a count of 5 $\log_{10}$ CFU/ml in 7 h with an initial inoculum of 1 $\log_{10}$ CFU/ml. However, in this study with chocolate milk, a viable count of 3.7 $\log_{10}$ CFU/ml in 2 d storage at 30°C was recorded with an initial inoculum of 3.3 $\log_{10}$ CFU/ml.

The same authors (Feijoo et al. 1997) also showed that germination and outgrowth of *B*. *cereus* spores in whipping cream resulted in predicted counts of 5 $\log_{10}$ CFU/ml at 32°C in 7 h with an initial inoculum level of 1 $\log_{10}$ CFU/ml. However, in our study, this increase was only marginal.

Considering the changes that could happen in the product due to the growth of *B*. *cereus* occurring either as vegetative cells and/or spores in chocolate milk, the increase in viable counts resulted in a decrease in pH of the product from an initial pH 6.4 to 4.5 in 6 d of storage, whereby the product attained characteristics of spoilage like off flavour and ropiness. It could be a possibility that once the product reaches certain pH level, the environment may become unfavourable for good growth



A



B

Figure 25 Assay plates showing positive lecithinase activity in experimental samples of *Ragi hurittu* [**A**] and chocolate milk [**B**]

of *B*. *cereus*. This may be one of the reasons for low population of *B*. *cereus* in chocolate milk, despite the spiking of this bacterial species at high initial numbers. As such, chocolate milk being a ready-to-drink product of slight viscous consistency and a good source of nutrients (proteins and carbohydrates) does offer an ideal environment for survival and growth of *B*. *cereus*.

On the other hand, *Ragi hurittu* being more of a dehydrated product profile, provides fewer opportunities for better survival and growth of *B*. *cereus*. Further, any increase in viable population did not necessarily bring in changes in sensory profile of the product. Even the pH level do not show any marked changes during storage of product. However, the bacterial species tend to remain in the product, whether introduced as vegetative cells and/or spores and subsequently, do increase in a slow paced manner. Such a product profile with no visual changes, but harbouring viable population of *B*. *cereus* is of high risk from the viewpoint of causing potential health hazards. The contrasting nature of product profile included in the present study gives an overview to assess the probable risk factors that could be associated through the behaviour in terms of growth of an equally opportunistic foodborne pathogen like *B*. *cereus*.

5.0 SUMMARY AND CONCLUSION

In the background of Indian scenario, the present study attempted to assess the prevalence of toxigenic traits among isolates of *B. cereus* in selected foods being sold in local markets based on biochemical and molecular characterization. Along with this, a phylogenetic relationship of the selected native isolates with those documented earlier in regard to their nucleotide sequence of selected toxin genes was also the focal point of study. In view of the ability of *B*. *cereus* to exist in dual phase and the majority of Indian traditional foods being heat processed, it was felt necessary to assess the thermal inactivation profile of native isolates of *B*. *cereus*. Another focal point of significance was to predict the behaviour of a selected toxigenic isolate in terms of two important phases of growth cycle. Also, the behavioural study with the same toxigenic isolate was extended to selected food matrices. The research findings of this study are summarized and also presented a thoughtful conclusion in the following paragraphs:

5.1 SUMMARY

5.1.1 Prevalence of potent toxigenic / pathogenic isolates of *Bacillus cereus* in Indian traditional foods

In a diverse range of foods including traditional fast foods using the selective polymyxin egg yolk mannitol bromothymol blue agar, the mean viable population of *B*. *cereus* ranged from a minimum of 3.4 $\log_{10}$ CFU/g in case of milk-based foods to a maximum of 5.1 $\log_{10}$ CFU/g in spice-based traditional fast foods. The percent incidence of *B. cereus* isolates in relation to total number of samples analyzed ranged from a highest of 40% in milk-based foods to a lowest of 10% in samples of processed wheat-based foods. In a total of 65 food samples, 26 isolates were characterized as *B*. *cereus* based on morphological, cultural and biochemical characteristics. A few of these isolates could grow at low and higher temperatures. These characterized isolates when subjected to

PCR with species specific primers of phosphatidyl inositol phospholipase C (Pi-PLC) revealed 46% (12 isolates out of 26) to be confirmed as *B*. *cereus*. Further, the identity of these selected isolates was confirmed by 16S rRNA primers. Of the PCR confirmed native isolates, 8 (67%) of them showed positive amplification with primers (Ha-1) of haemolysin BL gene (*hbl*). Only 50% of these *hbl* positive isolates exhibited discontinuous pattern of haemolysis in blood agar plates. In these native isolates, the primers designed targetted only the B component of haemolysis BL gene complex and there may be the absence of either one or both lytic components (L_1 and $L_{2)}$). Similarly, 50% of *B*. *cereus* isolates were positive for sphingomyelinase gene.

Molecular basis for the microbial diversity was studied by genetic analysis of four selected toxigenic native food isolates of *B. cereus* (CFR 1529, 1530, 1534 and 1536) based on the resultant partial nucleotide sequences of respective PCR amplicons of target genes (16S *rDNA*, *pi-plc, hbl and sph*) and degree of sequence homology. Force Type Neighbour Joining phylograms generated based on these sequences revealed high degree of homology (>90%) of the native isolates with other documented strains of *B. cereus* and species of *B. cereus* cluster. This showed that although the strains compared in the alignment were from diverse habitat and some belonging to different species altogether, a good degree agreement did exist between the sequences compared. At the same time, RAPD-PCR analysis of the 12 native isolates of *B*. *cereus* was undertaken to assess their genetic diversity. The total amplified products of the selected 4 random primers chosen was 168 (average of 42 bands per primer), which ranged from almost 100 to 1000 bp. The genetic similarity (GS) coefficient for the 12 isolates of *B. cereus* resulting from RAPD analysis ranged from 0.040 (between the isolates CFR 1534 and CFR 1533) to 0.470 (between the isolates CFR 1532 and CFR 1535), except CFR 1540. The dendrogram generated based on GS coefficients of RAPD analysis showed clear distinction into major and minor clusters with the presence of 3

major clusters A, B and C and sub-clusters within the major clusters. A hypothesis was proposed to explain the clustering pattern of the isolates of *B. cereus* based on the toxigenic traits present in the isolates, their psychrotophic growth profile and specific source of their isolation (food products). It was of interest to observe that isolates of *B. cereus* obtained from a specific type of traditional fast foods did harbour toxigenic traits in them and occurred under one cluster namely cluster A.

5.1.2 Thermal inactivation profile of *Bacillus cereus* vegetative cells and spores subjected to simulated time-temperature combinations of Indian traditional foods

The thermal inactivation profile in terms of *D*- and *z*-values of vegetative cells and spores of 3 potent toxigenic native food isolates of *B. cereus* were determined in saline, brain heart infusion (BHI) broth, skim milk and whole milk. The *D*-values for the vegetative cells of test isolates across the different menstra ranged from the lowest of 3.45 min at 60ºC to the highest of 10.6 min at 56ºC in saline. The findings revealed that there was no marked difference in the thermal resistance pattern of vegetative cells of the cultures studied. The mean *D*-values for saline, BHI broth, skim milk and whole milk were 5.6, 5.5, 5.4 and 5.1 min, respectively. The 12 *D*-values, also termed as commercial sterility values for vegetative cells of *B. cereus* cultures ranged from a lowest of 42 min at 58 and 60ºC to a highest of 127.2 min at 56ºC. The z-values of vegetative cells of *B. cereus* cultures ranged from 9.3°C for *B. cereus* CFR 1521 in BHI broth to 24°C for *B. cereus* CFR 1532 in whole milk. The results showed that heating media did not influence as a singular effect on heat inactivation pattern of vegetative cells. The thermal death curve for the vegetative cells in the present study was non-linear indicating that the inactivation rate was not constant, but rather exhibited a sigmoidal shape with shoulder and tailing pattern.

The *D*-values of spores of the same isolates ranged from a lowest of 4.4 min at 95°C to a highest of 19.45 min at 85ºC. Further, mean *D*-values obtained for the spores in different heating menstra like saline, BHI broth, skim milk, and whole milk were 9.6, 8.9, 7.3 and 9.6 min, respectively. The medium composition of heating menstra did not appear to have any marked influencing effect on the inactivation profile of the cultures studied. The survival curves obtained for spores showed a curvilinear pattern with shoulder (lag phase) followed by a linear declining pattern. It could be concluded that the inactivation pattern did not follow first order kinetics. The *z*-values of the spores of isolates of *B. cereus* ranged from 16.6 to 38.4°C.

The research leads from present study gave insight about the thermal inactivation pattern of native food isolates of *B*. *cereus* and could provide a baseline data to formulate time-temperature combinations during thermal processing of high complex food matrices.

5.1.3 Influence of cultural and nutritional attributes on the behavioural pattern of *Bacillus cereus* (vegetative cells and spores) in broth system

This study was focussed to predict the behaviour of vegetative cells and spores of a potent toxigenic native food isolate of *B*. *cereus* CFR 1534 in terms of lag phase duration (LPD) and growth rate (GR) as a function of temperature, pH and sodium chloride concentration in broth system. The experimental LPD values for vegetative cells ranged from 3.13 to 31.3 h. The lowest LPD was recorded under conditions of storage temperature of 30°C, pH of 5.5 and 4% NaCl, while the highest LPD value was at a temperature of 48°C, pH of 6.5 and 4% NaCl. A fairly high degree of correlation coefficient did exist between experimental and predicted values. Analysis of correlation coefficient showed that temperature had an influencing effect on the LPD values. In the case of studies relating to GR, the experimental GR values ranged from 0.2 to 2.2/h. The

lowest GR of 0.2 was recorded at a combination of 48°C, pH 6.5 and NaCl concentration of 4%, whereas the highest GR of 2.2 was with a combination of 30°C, pH 7.5 and 4% NaCl. The pH of medium was found to have the primary influence on GR of *B*. *cereus* CFR 1534.

The response surface plots for vegetative cells of *B*. *cereus* CFR 1534 revealed interesting behavioural pattern. At pH levels of 5.5 and 6.5, lower LPD values were observed with incubation temperatures of 30 and 36°C. At these two temperatures, the LPD values progressively decreased with increasing levels of NaCl from 2 to 6%. In contrast, at pH 7.5 with the same incubation temperatures, the LPD values were very low with negative values being observed initially at NaCl levels of 2 and 2.5%. However, increase in NaCl levels showed an increasing trend in LPD values. At the same time, GR values generated revealed a varied pattern at all the 3 pH levels of 5.5, 6.5 and 7.5. At pH levels of 5.5 and 6.5, the range of GR values derived resulted in categorization pf growth temperatures and NaCl levels. However, at pH 7.5, higher values ranging from a minimum of 1.3/h to a maximum of 2.0 to 2.5/h were visualized at 2 to 3 combinations of incubation temperatures and NaCl levels.

The experimental trials with spores of *B*. *cereus* CFR 1534 revealed that LPD ranged from 5.85 to 20.5 h. With the parameters of pH and NaCl being the same i.e. 6.5 and 4%, respectively, the lowest LPD was recorded at a storage temperature of 42°C and the highest at 22°C. Storage temperature appeared to be the primary influencing factor for the LPD of spores. On the other hand, GR values for spores ranged from 0.24 to 0.73/h, wherein the defined conditions of storage temperature, pH and per cent NaCl were the same as observed with GR of vegetative cells.

In the case of spores of *B*. *cereus* isolate, the behavioural pattern as could be visualized from response surface plots revealed that at pH levels of 5.5 and 6.5, lower LPD values were generated at temperatures of 34-42°C and NaCl levels of 2 to 3.5%. At

pH 7.5, the LPD values ranged from a minimum of 9.9 to a maximum of 21.4/h. At all the combinations of incubation temperatures and pH levels, the LPD values showed a progressive increase with increasing levels of NaCl. For the GR values with spores, the trend in behaviour was an increase from the initial GR levels at 2-3% NaCl levels and at incubation temperatures of 22-42°C, after which at levels of 3.5-6.0% NaCl, the GR values progressively decreased with the lowest value being with 6% NaCl. At pH 6.5, the increase from the initial GR values was observed at temperatures of 22 and 26°C and NaCl levels of 2-3.5%, after which the values progressively decreased. At pH 7.5, the GR values generated were of negative values at a temperature of 22°C with NaCl levels of 2-6%. A similar trend to that of pH 5.5 and 6.5 in respect of initial increase and subsequent decrease was observed at the temperatures included in the experimental design, except for 22°C.

The study provided certain leads towards assessing the quantitative effect and synergistic interaction of the three influencing factors (temperature, pH and salt concentration) on the LPD and GR of a selected native isolate of *B*. *cereus* CFR 1534. The results may in all probability enable to predict the behaviour of an opportunistic foodborne pathogenic bacterial species like that of *B*. *cereus* under defined conditions, which in turn would have a practical application in the commercial food chain.

5.1.4 Behavioural pattern of *Bacillus cereus* in selected food matrices

In contrast to the study attempted in the previous experimental chapter with broth system, in the present experimental trials, an attempt was made to assess the behaviour of vegetative cells and spores of a native potent toxigenic food isolate of *B*. *cereus* CFR 1534 in two different food matrices, wherein one was a cocoa-based beverage of reasonable total solids and the other a nearly dehydrated traditional nutrient rich millet and legume-based food product.

5.1.4.1 Ragi hurittu

In the dehydrated powdered traditional product based on finger millet and Bengal gram flour commonly known in the vernacular language as *Ragi hurittu*, at storage temperatures of 20 and 30°C and initial inoculum of 3.3 $\log_{10}$ CFU/g of vegetative cells of *B*. *cereus*, there was an increase of almost 2.0 logs in the viable count in 2 d of storage, after which the numbers remained same even till 6 d of storage. With initial levels of 5.3 and 7.3 $\log_{10}$ CFU/g, there was no appreciable increase observed during storage period. Similarly, at storage temperature of 40°C, there was no appreciable change in the viable counts from the initial levels introduced in to the product. However, with initial level of 7.3 $\log_{10}$ CFU/g and at the 3 storage temperatures, there was a marginal decrease in the viable population of *B*. *cereus*, and this decrease was quite appreciable in 6 d of storage at 40°C. A reasonable degree of correlation coefficient was observed between experimental and predicted values. In the case of spores of *B*. *cereus* CFR 1534, at the defined storage temperatures, no changes were observed from that of the initial spiked inoculum level of 3.3 $\log_{10}$ CFU/g. However, with initial levels of 5.3 and 7.3 $\log_{10}$ CFU/g, there was reduction in the viable numbers during storage period. A high degree of correlation between experimental and predicted values suggested a good model fit.

The response surface plots generated for vegetative cells revealed that in 2 d of storage at the selected storage temperatures for RSM, the viable count of *B*. *cereus* revealed a marginal increase of 1-2 logs from the initial level of 4.3 $\log_{10}$ CFU/g. Subsequently, the counts had a slow paced decreasing pattern during 3-6 days of storage. A similar trend was also observed with the inoculums level of 6.3 $\log_{10}$ CFU/g, wherein the initial increase in viable count was less than 1.0 log in 2 d. In the case of spores, irrespective of the initial inoculum levels, at the selected storage temperatures, the trend was of a decreasing pattern right from 2 days of storage, wherein at least 1 log reduction in counts was observed in a storage period of 10 d.

5.1.4.2 Chocolate milk

As against a traditional dry product of low water activity, in another set of experimental trials, the behaviour of vegetative cells of the same potent toxigenic native isolate of *B. cereus* CFR 1534 was studied in chocolate milk, which was of slight viscous in nature. The vegetative cells as well as spores of *B. cereus* introduced at all the 3 initial inoculums levels and storage temperatures revealed a marginal increase in viable population which more or less reached 1 log in 6 d of storage. A high degree of correlation coefficient was observed between experimental and predicted values and in both the cases of vegetative cells and spores, the primary influencing factor was the level of initial inoculum. The response surface plots generated for vegetative cells revealed a similar pattern to that observed in the experimental trials, wherein the surface plots clearly presented a slow paced increase in viable populations during the storage period reaching 1 log increase in 6 d. The response plots for spores showed a marginal decrease in counts in the initial 2 d storage at 20-40°C from those levels of spores spiked in to the product. Subsequently, a slow paced increasing pattern was visualized till 6 d of storage increasing by 1 log population.

During the above changing pattern in the profile of both vegetative cells and spores *B. cereus* in chocolate milk, the pH of the product decreased from an initial level of 6.4 to 4.5 during storage period of 6 d. This led to higher acidity accompanied by off odour and slight ropiness in the product indicating signs of microbial spoilage. Under any given situation, the survival and growth of the organism under question would be more affected by the product profile and the change in environmental factors. This could lead to a situation of risk associated factors due to the toxigenic traits being harboured in the bacterial species like *B. cereus*, the one included in this study.

5.2 CONCLUSION

The research leads revealed that isolates of *B*. *cereus* were present in most of the foods being marketed in the local market. The viable population of this bacterial species varied based on the profile of food and the extent of hygienic and sanitary practices being in operation at all stages of food chain for this specific product. Being an opportunistic pathogen, it is quite likely that on many occasions the toxigenic traits being harboured in the isolates could express under given cultural and environmental factors. Such a situation would make the food to be a cause of health hazards and thus become unsafe for human consumption. The ability of *B*. *cereus* to occur in the dual phase of vegetative cells and spores under changing intrinsic and extrinsic factors provides the added advantage for its survival against varied process parameters targeted towards destruction of bacterial organisms. It is for this reason that the survival profile is so varied, wherein it becomes highly complicated to eliminate *B*. *cereus* uniformly in all the food environments, as it was amply established in two different food matrices in the present study.

The microbial diversity could be well visualized through the application of molecular biology methods, wherein almost 90% homology does exist in the genetic make-up of the strains of *B*. *cereus* as well as there occurs a close relationship with other species of the cluster. The phenotypic and genotypic traits of the bacterial species have been of evolutionary origin and are spread across the strains uniformly with minor variations, irrespective of their habitats and geographical identities.

The understanding of aspects relating to health hazards through microbial food safety is a continuing knowledge base generation programme that would enable to build-in the concepts of Good Manufacturing and Distribution Practices (GMDPs), Good Hygienic Practices (GHPs) and Hazard Analysis Critical Control Point (HACCP) in the food chain to achieve safe and healthy foods to the human population.

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